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Detection and management of Colletotrichum acutatum sensu lato on strawberry

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Detection and management of *Colletotrichum acutatum sensu lato* on strawberry

by

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DEDICATION

To my Father Mr. Donghong Zhang, who always encourages me to pursue my dream and shows me a great example of hard working and my Mother Mrs. Hua Zhao, who gives me love and attention that make me brave.

To my husband Mr. Gang Han, who is supportive and optimistic like always, my lovely daughter Muxi Mercy Han, who was born during my Ph. D. study and makes my life to be more meaningful and my dearest Lord, who loves me and gives me all the precious things.

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ABSTRACT

Colletotrichum acutatum sensu lato, one of the most economically damaging pathogens of strawberry, is the primary causal agent of anthracnose fruit rot (AFR). A key challenge in managing AFR is detecting the pathogen on asymptomatic plants. To meet this need, a loop-mediated isothermal amplification (LAMP) assay was developed that incorporated two sets of primers: LITSG1 targeted on the ITS region of ribosomal DNA and Ltub2 on the β-tubulin 2 gene. In pure culture assays, Ltub2 was specific for detection of *C. acutatum*, whereas LITSG1 detected *C. acutatum* and two additional anthracnose pathogens, *C. gloeosporioides* and *C. fragariae*. LITSG1 had 10-fold higher sensitivity (20 pg of mycelial DNA) than Ltub2 (200 pg) in detection of *C. acutatum* from pure cultures. The LAMP assay was also tested on asymptomatic greenhouse and field plants, and was shown to have strong potential for detection of *C. acutatum* in planta.

Field experiments were conducted at the ISU Horticulture Research Station near Gilbert, IA, during the 2012, 2013, and 2014 growing seasons to validate an AFR warning system that had been previously developed and tested in Florida. Five treatments included a factorial combination of two spray timing methods (warning system and calendar-based) and two fungicides (captan and pyraclostrobin), plus a non-sprayed control. The day-neutral strawberry cultivar Tristar was spray-inoculated with *C. acutatum* conidia at the beginning of the bloom period. In each year, the AFR warning system saved one to two fungicide sprays compared to calendar-based treatments. In general, the warning system-based treatments controlled AFR as well as calendar-based sprays. The results provide evidence that the Florida warning system may be valuable for helping Midwest strawberry growers to reduce fungicide use against AFR.

Finally, I developed a case study entitled “Strawberry anthracnose: managing a hidden menace” to challenge students to help an Iowa strawberry grower decide how to manage AFR with fewer fungicide sprays. When students study this case, they learn how plants become infected and how a disease-warning system uses information about the weather to help growers manage diseases with less reliance on fungicides. The case study was tested by Iowa State University horticulture and plant pathology undergraduate students, and feedback from students and instructors has been integrated to improve the case study.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is comprised of one abstract and five chapters. The first chapter provides an introduction, literature review and research objectives. Chapter two describes a loop-mediated isothermal amplification (LAMP) detection method for the fungal pathogen *Colletotrichum acutatum sensu lato* on strawberry. Chapter three reports results from a three-year field study in Iowa to validate a warning system for anthracnose fruit rot that was originally developed for use by strawberry growers in Florida. Chapter four presents an online educational case study for undergraduate-level courses on disease management of *C. acutatum sensu lato* on strawberry. Finally, chapter five contains a summary of the results and conclusions of this dissertation.

Literature Review

The disease

Anthracnose fruit rot of strawberry

Garden strawberry (*Fragaria×ananassa*) arose from the hybridization of the North American species *F. virginiana* (octoploid, 2n=56 chromosomes) and the South American species *F. chiloensis* in the 1700s. Because of its attractive flavor and adaptable nature, it has become an important fruit crop worldwide (Howard et al., 1992). The United States is one of the top three strawberry-producing countries worldwide. In 2010, approximately 58,070 acres of strawberries

were planted in the U.S. with a value of $2.26 billion (USDA, 2010). However, pests and diseases reduce the quality and yield of strawberry fruit, and impose severe economic losses on commercial growers.

Anthracnose attacks many parts of a strawberry plant, results in crop losses that can exceed 50%, even in well-managed fields (Turechek et al., 2006). Three principal pathogens are traditionally believed to cause strawberry anthracnose: *Colletotrichum acutatum* J.H. Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. (teleomorph *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk) (Howard et al., 1992; Smith and Black, 1990). Of these, *C. acutatum* is the most prevalent fruit-rotting pathogen (Freeman et al., 1998). In recent studies, *C. acutatum* and *C. gloeosporioides* have been reclassified as species complexes, and many new species have been described within these two complexes (Cannon et al., 2012). However, the primary basis for creating these new species designations was genetic information; therefore, more intensive studies are needed to identify their biological characteristics and host-pathogen relationships (Cannon et al., 2012; Damm et al., 2012; Harrington and Rizzo, 1999). In this dissertation I will use the name *C. acutatum sensu lato* to designate a species complex, in recognition of the continued uncertainty surrounding the validity of the more recent splits of the complexes into multiple species. In addition to the three principal pathogens, *C. dematium* has also been reported as a pathogen of AFR (Smith and Black, 1990).

C. acutatum sensu lato can attack almost all parts of the plant (Arroyo et al., 2005; Peres et al., 2005). Disease symptoms on mature fruit are firm, sunken lesions that are brown or black in color. The lesions may enlarge until they cover the entire fruit (Howard et al., 1992). Pathogen-infested or -infected nursery plants are the most important source of primary inoculum in many production fields (Prusky et al., 2000), in part because the pathogen can survive long periods on

host tissue in a quiescent condition, invisible to the naked eye. Furthermore, epiphytic *C. acutatum sensu lato* can form secondary conidia without showing symptoms, and these conidia can then spread within a field by means of splashing water (Leandro et al., 2001). As a result, AFR is challenging to manage, because symptomless infected and infested plants escape inspection and the pathogen can build up to high levels in the field without being detected, setting the stage for severe epidemics on ripening fruit under disease-favorable weather conditions. The pathogen can over-winter (in the northern U.S.) or over-summer (in Florida) in soil or within plant debris. Alternative host plants also serve as sources of primary inoculum (Freeman et al., 2001; MacKenzie et al., 2009). Annual production fields are often fumigated before planting to minimize over-seasoning inoculum.

Symptoms of strawberry anthracnose

Anthracnose caused by *Colletotrichum* species impacts not only fruit but also crowns, petioles, leaves, buds, and flower parts. This versatility, and the fact that several *Colletotrichum* species are involved, helps to explain the complex nature of the disease (Howard et al., 1992). The term "anthracnose" was first used to describe the dark-brown oval lesions on strawberry runners (occasionally on petioles) caused by *C. fragariae* in Florida (Brooks, 1931). Later, "strawberry anthracnose" described all symptoms of strawberry caused by *Colletotrichum* species. However, the various species causing anthracnose cannot be distinguished in the field by symptoms alone (Curry et al., 2002; Howard et al., 1992).

Fruit rot: This symptom initially appears similar to sunburn damage, forming light brown, water-soaked spots on fruit during ripening. Soon, however, the characteristic firm, sunken, rounded, and dark brown to black lesions develop. When plants are wet for prolonged periods during warm weather, the lesions will develop an orange-pink cast due to production of masses

of conidia in a slimy matrix of extracellular polysaccharide. In some production regions, lesions can develop on unripe (green) fruit. These lesions are firm and small with dark brown to black color, and will eventually develop into characteristic ripe-fruit lesions (Howard et al., 1992).

Flower blight: Before flower buds open, the sepals and pedicels can be infected. Affected buds typically dry up and turn brown. When open flowers are infected, they develop dark lesions that extend to the pedicel of the infected calyx, turn brown, and quickly dry out. Small, deformed fruit will appear if flower infection occurs after pollination.

Irregular leaf spot: Dry, dark brown to black lesions with irregular borders develop along leaf margins and tips. This symptom is similar to those caused by fertilizer burn or physiological stress, but an incorrect diagnosis can place a field at risk of a subsequent fruit rot or crown rot outbreak.

Lesions on stolon and petioles: These are sunken, dark dry spots with clear boundaries. The lesion will eventually girdle the stems and then wilt the subtending leaves and daughter plants.

Crown rot: The most common symptom caused by *C. fragariae* is crown rot. However, *C. acutatum sensu lato* also causes crown rot as a result of the dissemination of spores originating from other plant parts. When crown rot severity reaches a sufficiently high level, the plant will suddenly wilt and die. A reddish-brown discoloration of the vascular system can be seen when infected crowns are split open longitudinally (Freeman and Katan, 1997).

The pathogen

The genus *Colletotrichum*

The genus *Colletotrichum* includes many important plant pathogens that cause disease in a wide range of hosts. The geographical ranges of these pathogens are primarily in tropical and subtropical regions, but some important crops in temperate regions also can be infected, including strawberry, citrus and avocado (Cannon et al., 2012). Some economically important species have been well studied genetically as model organisms, including *C. graminicola, C. higginsianum,* and *C. orbiculare* (Asakura et al., 2009; Crouch and Beirn, 2009; O'Connell et al., 2012). Whole-genome sequences are available for *C. graminicola, C. higginsianum*, and *C. fioriniae* (a newly described species in the *C. acutatum* species complex) (O'Connell et al., 2012; Baroncelli et al., 2014).

The taxonomy of species in the genus *Colletotrichum* is confusing due to their broad host ranges and varied lifestyles. *Colletotrichum* names in current use include a total of 66 common species, with an additional 19 recently used names that remain controversial (Hyde et al., 2009). However, some formerly designated species were considered to be species complexes, and new species continue to be recognized. Among the nine species complexes in the genus, *C. acutatum sensu lato* and *C. gloeosproioides sensu lato* have been recognized as two major clades on a multilocus phylogenetic tree (Cannon et al., 2012).

Colletotrichum acutatum sensu lato

Taxonomy: *Colletotrichum acutatum* J. H. Simmonds was first described as a distinct species of fruit-rot pathogen in Queensland, Australia (Simmonds, 1965, 1968). Previously, the pathogen was described as a *Gloeosporium* species that caused ripe fruit rot and stolon, petiole, and

peduncle lesions on strawberry in Australia (Arx, 1970). *C. acutatum sensu lato* belongs to kingdom Fungi, subkingdom Dikarya, phylum Ascomycota, subphylum Pezizomycotina, class Sordariomycetes, subclass Hypocreomycetidae, order Glomerellales, family Glomerellaceae and genus *Colletotrichum*. Perithecia were first observed on artificial culture in sexual compatibility studies (Guerber and Correll, 1997) and the teleomorph, *Glomerella acutata,* was identified as the pathogen causing bitter rot of apple (Guerber and Correll, 2001). Within the *C. acutatum* species complex, only *C. salicis* and *C. rhombiforme* formed sexual structures under laboratory conditions. Twenty-eight additional species were recently named that also belong to the *C. acutatum* species complex, but currently lack described teleomorph stages (Damm et al., 2012).

Description: *C. acutatum sensu lato* typically has hyaline, smooth-walled, septate, branched hyphae, 1-5.5 μm in diameter. Conidia are hyaline, aseptate, straight and smooth, fusiform to cylindrical, with one or two acute end(s). Sizes of conidia vary considerably among different species of the *C. acutatum* complex: length × width (μm^2) varied from 6.7 × 4.1 μm^2 to 22.3 × 4.5 μm^2 (Damm et al., 2012). Conidiophores are hyaline, smooth-walled and mostly simple. On the host, conidia are produced in acervuli as pink or orange masses. Setae are rarely observed; if present, they are dark brown, tapered and thick-walled, without producing conidia like some other *Colletotrichum* species (Lenné et al., 1984). Appressoria are solitary, light to medium brown, smooth-walled, clavate to obovate and borne on undifferentiated hyphae. Colonies on potato-dextrose agar (PDA) are white, pink orange in color, and will turn gray or black with age. From the underside of culture plates, the colonies are orange or salmon with streaks of gray or black (Damm et al., 2012).

Host range and world distribution: *C. acutatum* has a wide host range and a worldwide distribution. Hosts include woody and herbaceous crops, ornamentals, fruits, and conifers.

Strawberry, grape, blueberry, lupine, almond, citrus, apple, olive, peach and pine are among the hosts that can experience economically significant losses (Peres et al., 2005; Sreenivasaprasad and Talhinhas, 2005).

Additional species in the strawberry anthracnose complex

C. fragariae was the first fungus identified as an anthracnose pathogen in Florida (Brooks, 1931). It spread throughout the southeastern United States and was responsible for crown rot and death of many nursery plants in the 1970s (Smith, 2008). Although *C. fragariae* can cause stolon and fruit lesions as well as summer wilt, it is more often associated with severe petiole and crown symptoms than *C. acutatum sensu lato* in warm, humid areas such as Florida (Curry et al., 2002). The host range of *C. fragariae* is not as broad as *C. acutatum sensu lato* and *C. gloeosporioides sensu lato;* it is limited to strawberries, several weed species, silver date palm and cyclamen (Mackenzie et al., 2008). *C. fragariae* is found mainly in Florida as well as in strawberry nurseries in California, the Northeast U.S., and Nova Scotia and Ontario in Canada (Howard et al., 1992).

C. gloeosporioides sensu lato can cause anthracnose crown rot, petiole lesions, and leaf spots on strawberry, but is responsible primarily for crown rot (Mackenzie et al., 2007). The spore morphology and disease symptoms of *C. gloeosporioides sensu lato* are very similar to those of *C. acutatum sensu lato,* so it is difficult to distinguish them in the field. Conidial shape is commonly used to identify *C. gloeosporioides sensu lato*; it is typically cylindrical with rounded ends. The teleomorph, *Glomerella cingulata,* occurs widely and generally produces olive or dark grey colonies on PDA (Smith and Black, 1990). *C. gloeosporioides sensu lato* was recently reclassified as 22 species and one subspecies; these distinctions were made primarily on the basis of DNA sequence differences (Weir et al., 2012). The host range of *C. gloeosporioides sensu*

lato is very wide, including food crops like strawberry, banana, papaya, grape, citrus, mango, coffee and tea as well as ornamental crops and weeds (Weir et al., 2012). The ability of *C. gloeosporioides sensu lato* to invade strawberry fields from plant species on field edges was confirmed in Florida (Mackenzie et al., 2007).

Genetic diversity of *Colletotrichum acutatum sensu lato*

As an economically important pathogen with a wide host and geographic range, *C. acutatum sensu lato* is also morphologically and genetically diverse. The current debate over classifying this pathogen as a single heterogeneous species or multiple species focuses mainly on whether genetic differences are sufficient to delineate species in the absence of additional morphological or physiological evidence (Hyde et al., 2009; KoKo et al., 2011). In 2005, eight *C. acutatum sensu lato* subgroups were delineated based on analysis of ITS sequences (Sreenivasaprasad and Talhinhas, 2005). More recently, *C. acutatum sensu lato* was disaggregated into 31 species based on phylogenetic analysis of six genes coupled with morphological evidence (Damm et al., 2012).

Diversity of *Colletotrichum* species on strawberries was first explored in Israel using vegetative compatibility grouping and arbitrarily primed PCR (ap-PCR) (Freeman and Katan, 1997). Isolates of *C. acutatum* in the same VCG group had nearly the same ap-PCR band pattern as *C. acutatum* in the U.S., in contrast to *C. fragariae* from the U.S. and Canada. Although researchers suggested that all of the *C. acutatum sensu lato* isolates in the study had been introduced from a single strain in the U.S., they did not make comparisons with isolates from other regions. The same research group later delineated four subgroups within *C. acutatum* from several hosts and geographic origins using ap-PCR, A+T-rich DNA analyses with ITS-2 sequence analysis, and analysis of the complete ITS region (Freeman et al., 2001). All three

methods grouped the isolates into four groups, and isolates within each group lacked host specificity.

A subsequent study employed random amplified polymorphic DNA (RAPD) combined with ITS 2 sequence data to divide *C. acutatum sensu lato* into two subgroups: CA-clonal and CA-variable (Denoyes-Rothan et al., 2003). The CA-clonal subgroup had identical RAPD and nearly identical ITS2 sequences, was prevalent in Europe, and contained isolates only from strawberry. The CA-variable subgroup exhibited variable RAPD patterns and divergent ITS2 sequences, and included isolates from various hosts. These authors hypothesized that isolates in the CA-clonal subgroup might develop host specialization on strawberry.

A diverse collection of fruit and foliar *C. acutatum sensu lato* isolates from a wide range of hosts and geographic regions was characterized using random amplified polymorphisms (RFLPs) of mtDNA, as well as RFLPs plus sequence analysis of introns of glutamine synthetase (GS) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes (Guerber et al., 2003). Seven distinct molecular groups were reported, as well as mating compatibility within clade C and between clades C and J4. Clade C had isolates from a wide range of hosts and geographic origins and J4 contained isolates from Australia and New Zealand, recovered from fruit rot and pine seedlings with terminal crook disease. Based on mating compatibility, Grueber et al. suggested that genetic isolation had occurred prior to reproductive isolation. Later, Damm et al., using six genes - the 5.8S nuclear ribosomal gene with the two flanking ITS, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), partial sequences of the chitin synthase 1 (CHS-1), histone3 (HIS3), actin (ACT) and beta-tubulin (TUB2) - built up a phylogenetic tree and took a further step by disaggregating *C. acutatum sensu lato* into 31 species (Damm et al., 2012). In their collection, 5 species caused AFR on strawberry: *C. nymphaeae, C. simmondsii, C. fioriniae, C. codetiae* and

C. salicis. Among these species, only *C. salicis* produced perithecia under lab and natural conditions.

Assays for detection

Importance of detection

Conventional methods for fungal pathogen detection rely on disease symptom diagnosis, biochemical tests, pathogenicity tests, pathogen isolation, and morphological identification (Atkins and Clark, 2004). For *C. acutatum sensu lato* detection on strawberry, however, pathogen detection and isolation usually require a 5- to 7-day incubation period (Mertely and Legard, 2004). Additional complicating factors with traditional methods include the need for experienced, skilled laboratory staff, because of the morphological similarity of key *Colletotrichum* species.

For effective management of strawberry AFR, these limitations of traditional diagnostic methods pose challenges. One reason is that the long period required for diagnosis requires an extended quarantine if nursery plants are being tested. The pathogen can infect and survive for extended periods in a symptomless, quiescent stage on strawberry plants, so early detection of *C. acutatum sensu lato* in the field is also important. For detection of the pathogens in strawberry nurseries and production fields, a method needs to be sensitive, selective, and robust to widely varying field conditions. It also needs to be rapid, since anthracnose epidemics can spread very quickly.

Alternative detection methods

In order to shorten the detection time and increase sensitivity, several molecular-based detection methods for *C. acutatum sensu lato* have been developed.

The first PCR-based assay for *C. acutatum* (Sreenivasaprasad et al., 1996) used the specific primer CaInt2 (5'- GGGGAAGCCTCTCGCGG-3'), located on the variable internal transcribed spacer (ITS1) region of ribosomal DNA, with the universal fungal ITS primer ITS4; together, they enabled detection of *C. acutatum* from strawberry by amplifying a 490-bp DNA fragment. The method was also used to detect latent infection of *C. acutatum* in asymptomatic strawberry tissues (Parikka and Lemmetty, 2004). However, this primer set sometimes amplified a 650-bp nonspecific band, which was not the target of the amplification.

To encompass the high level of genetic diversity within *C. acutatum sensu lato*, another set of PCR primers, acut1 (5'-CCGGAGGAAACCAAACTCTATTTA C-3') and col2: (5'-TTACT ACGCAAAGGAGGCT-3'), on the ITS region was developed that were more specific than the earlier primer set (Martinez-Culebras et al*.,* 2003).

In order to achieve higher sensitivity for detecting *C. acutatum* on asymptomatic plants, a nested PCR assay was developed (Pérez-Hernández et al., 2008). This method used the general fungal primer set ITS1-F/ITS4 in the first round of amplification and the specific primer set CaInt2/ITS4 in the second round. It detected 1.0 fg of DNA extracted from mycelium, one infested leaf in 50 non-infested leaves in greenhouse trials, and asymptomatic but infected strawberry leaves in the field. Despite its 100-fold greater sensitivity than conventional PCR, this type of assay is more complex and time-consuming, and is prone to interference by contamination and cross-reactions from inhibitors.

A real-time PCR method was developed to meet the needs of detecting *C. acutatum sensu lato* specifically, sensitively and quantitatively in plant material (Debode et al., 2009). This method initially designed two primer sets - CaITS_F701/R699 (5′-GGATCATTACTGAGTTAC CGC-3′ and 5′-GCCCGCGAGAGGCTTC-3′), as well as CaTub_F430/R431 (5′-CGTCTACTT

CAACGAAGTTTGTTATCC-3′ and 5′-GAGGCCTGGTTGGGTGAG-3′) - targeted on the rDNA ITS1 region and the β-tubulin 2 gene, respectively. The ITS-based assay reliably detected 50 fg genomic DNA or 25 conidia, but the β-tubulin-based assay was approximately 66 times less sensitive. Although the sensitivity of real-time PCR was less than nested PCR, it required only one round of amplification compared to several for nested PCR, had no post-PCR processing, and provided quantitative data on the amount of pathogen DNA. These advantages made real-time PCR a powerful tool for studying the dynamics of the pathogen on its host. However, real-time PCR is not suitable for non-laboratory-based detection because it requires expensive instrumentation as well as technicians with special training.

Detection via PCR relies on methods for extracting DNA, verifying its purity, instruments that can perform fast thermal cycling, and gel electrophoresis to visualize the results. It also has low amplification efficiency, sensitivity and specificity compared to LAMP (Notomi et al., 2000). When samples show weak or nonspecific bands, detection results can be difficult to interpret. Therefore, trained technicians are also critical for conducting PCR assays. These limitations can pose a disadvantage when conducting screening of numerous samples for presence of the anthracnose pathogens on nursery plantlets or plants in a production field.

In addition to PCR-based methods, fingerprinting techniques such as restriction fragment length polymorphisms (RFLP) have been reported for *C. acutatum sensu lato*. The RFLP method used primer set GSF1/ GSR1, amplified a 1-kb intron of the glutamine synthetase (GS) gene intron region, and then digested the PCR product with restriction enzyme *Pst* (Liu et al., 2011). The results were phylogenetically informative and revealed higher variation than either the ITS region or the beta-tubulin gene.

LAMP detection

Loop-mediated isothermal amplification (LAMP) is a relatively simple, rapid, and low-cost method for genomic DNA detection, in part because it can amplify target genes under isothermal conditions, with no need for a thermal cycler (Notomi et al., 2000; Mori et al., 2009). The method employs a DNA polymerase (*Bst* DNA polymerase large fragment with high strand displacement activity), which originates from the soilborne bacterium *Geobacillus stearothermophilus.* It has strand displacement activity that can displace and release a third single-stranded new DNA during primer-initiated polymerization (Niessen and Vogel, 2010).

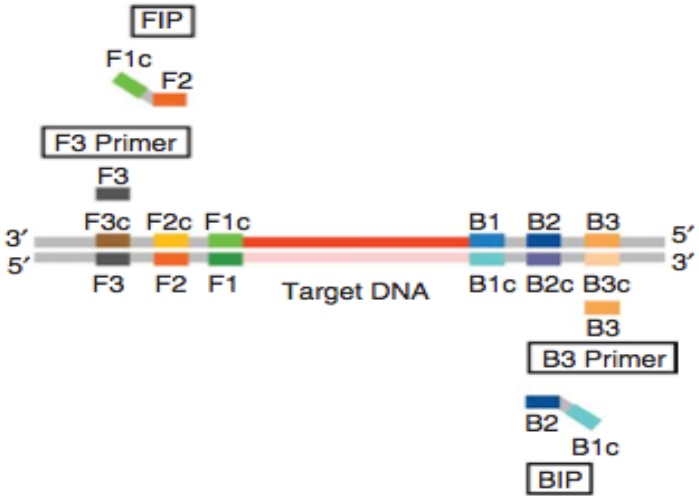

Figure 1. Primer location of the LAMP reaction
(Tomita et al., 2008)

The original LAMP assay (Notomi et al., 2000) used a set of four primers (two inner primers and two outer primers) with six binding sites on different regions of a target gene (Fig. 1). In order to explain the LAMP mechanism, six distinct target regions are labeled as F3, F2, F1, B1c, B2c and B3, starting from the 5' end of the target DNA. Sequences on the complementary strand are all labeled with c; for example, the F1c sequence is complementary to the F1 sequence. Two

inner primers (FIP and BIP) and two outer primers (F3 and B3) are used in the LAMP method. FIP and BIP are hybrid primers consisting of the F1c and F2, and B1c and B2 sequences, respectively.

When the target DNA and reagents are incubated at a constant temperature between 60-65°C, which is optimal for activity of the *Bst* DNA polymerase, the following two reaction steps proceed (Tomita et al., 2008):

Starting loop structure producing steps (structures 1-5 in Figure 2):

One inner primer (FIP) anneals itself to the complementary sequence of double-stranded target DNA when the target DNA is in a dynamic equilibrium condition at <65 °C. The *Bst* DNA polymerase initiates DNA synthesis and displaces single-stranded DNA. Then the F3 primer anneals to the F3c region and releases the FIP-linked complementary strand (step 1 to 3). The released single strand will then form a stem-loop structure at 5' end showed in step 4, because of the complementary F1c and F1 regions. The single strand DNA in step 3 serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement, in the same manner described for the FIP and F3 end. The structure generated by the BIP primer forms a loop structure at both ends (dumbbell-

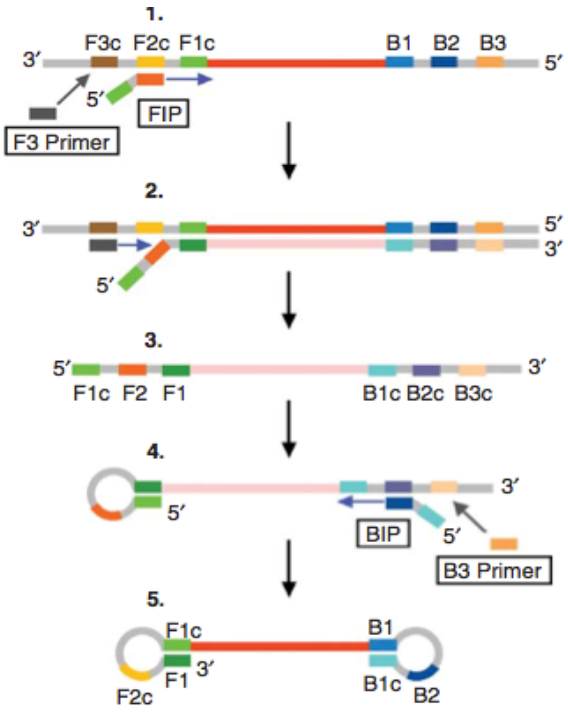

Figure 2. Formation of Loop structure (Tomita et al., 2008)

like structure) when the B3 primer anneals to the B3c region and releases the BIP-linked complementary strand.

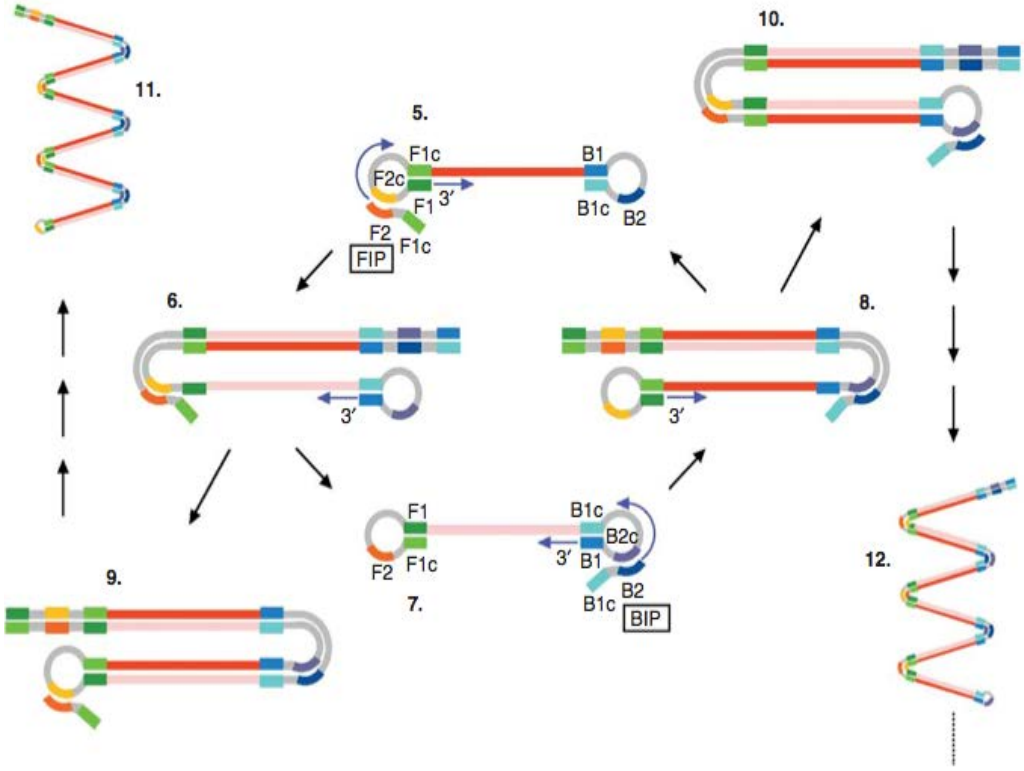

Figure 3. Cycling amplification (Tomita et al., 2008)

Cycling amplification steps:

Using structure 5 as a template, self-primed DNA synthesis is initiated from the 3' end F1 region, and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure. Passing through several steps, structure 7 is generated, which is complementary to structure 5. Later, structure 5 is produced from structure 8 in a reaction similar to that of structures 5–7. Finally structures 9 and 10 are produced from structures 6 and 8, respectively, and more elongated structures (11, 12) are also produced. (Mori and Notomi, 2009)

In order to accelerate amplification rate and thus reduce detection time, an additional two primers (Loop primers) were designed and added (Nagamine et al., 2002). Loop primers hybridize to the stem-loops that are not occupied by inner primers and primary strand displacement DNA synthesis. This improvement reduced the total time required for a LAMP analysis, including detection, to <1 h.

Plant pathogens of economic importance that have been detected by LAMP assays include *Fusarium graminearum*, the major causal agent of *Fusarium* head blight of small cereals and a producer of several mycotoxins. LAMP primers were designed on the galactose oxidase (gaoA) gene of the fungus and reaction results were visualized by calcein fluorescence. This assay detected the presence of less than 2 pg of purified target DNA per reaction within 30 min and was useful in identification of fungal isolates and detection of *F. graminearum* in total genomic DNA isolated from bulk samples of ground wheat grains (Niessen and Vogel, 2010). A LAMP assay for the ubiquitous plant pathogen *Botrytis cinerea*, targeted on the ITS regions of ribosomal DNA (rDNA), consistently amplified 65 pg DNA and had no cross-reactivity with a range of other fungal pathogens (Tomlinson et al., 2010a). With a novel real-time LAMP platform (the OptiGene Genie I), the assay amplified *B. cinerea* in infected rose petals in <15 min. As another example, a LAMP assay detected the woody plant pathogen *Phytophthora ramorum* in 45 min using only a heat block (dry bath block), and the LAMP product was identified by a color change (Tomlinson et al., 2007). This method was improved subsequently by developing easier and faster DNA extraction methods and adapting LAMP detection to a lateral-flow-device that could be adapted for non-laboratory use (Tomlinson et al., 2010b). LAMP assays have been also developed for several bacterial pathogens of economic importance on crop plants, including *Ralstonia solanacearum*, *Erwinia amylovora,* and *Xylella fastidiosa*. In

all these tests, LAMP showed its advantages as a simple, robust, efficient and inexpensive assay with adequate sensitivity and specificity for in-field detection applications (Bühlmann et al., 2012; Harper et al., 2010; Kubota et al., 2008).

Several factors can impact the effectiveness of LAMP detection. As mentioned above, the addition of loop primers can increase speed of detection (Nagamine et al., 2002). The reagent *Bst* DNA polymerase is vital to the assay, but needs to be mixed in gently and the incubation temperature should be between 60 and 65°C (Tomita et al., 2008). The Mg^{2+} concentration of the reaction has been reported to influence sensitivity of LAMP detection by impacting primer annealing and DNA polymerase activity (Yeh et al., 2005). As a by-product of the amplification, magnesium pyrophosphate can also be an indicator for visualizing the result of the amplification (Mori et al., 2001).

Contamination control for LAMP assay is essential. The amplification has high efficiency and the reaction can accumulate 10^9 copies of target in >1 hr. The final products are very long stem-loop DNAs with several inverted repeats of the target DNA (Notomi et al., 2000), which means if the final products get into the pipette, tubes or surrounding environment they will be amplified very easily by later reactions. Therefore, when handling LAMP-amplified product, opening and closing of the reaction tube should be conducted in a different room from where reagents and reaction mixtures are prepared (Tomita et al., 2008).

Disease-warning system for AFR on strawberry

Introduction

Disease-warning systems are tools to optimize the timing of plant disease management practices. They utilize information about one or more component of the disease triangle (weather, crop, and/or pathogen) to predict the risk of outbreaks or changes in intensity of one or more diseases (Campbell and Madden, 1990). Disease-warning systems for plant pathogens estimate the risk of epidemic occurrence and recommend spray applications only when environmental conditions are favorable for disease development.

There are two basic approaches to development of a disease-warning system: fundamental and empirical. Infection models created by the fundamental approach describe the infection response in relation to environmental parameters by using data collected from experiments in the laboratory and environmental chambers. In contrast, the empirical approach derives qualitative rules or quantitative models based on statistical relationships between summarized environmental inputs and disease observations in the field (Madden and Ellis, 1988; Ellis and Madden 1993). Compared to the empirical approach, the fundamental approach is more likely to be generic, standardized and adapted to different circumstances. In research on AFR conducted at Ohio State University, for example, the fundamental approach was used to create an AFR infection model, based on results of experiments conducted under controlled conditions (Wilson et al., 1990).

Temperature and moisture often impact the risk of epidemic development. Temperature influences all phases of pathogen development whereas the duration of wet periods, often described in terms of leaf wetness duration (LWD), influences the infection process (Huber and Gillespie, 1992; Leandro et al., 2003). Infection potential may also be related to other parts of the

disease cycle (Xia et al., 2007). Prolonged periods of high relative humidity may be required for sporulation and infection by certain plant pathogens (Colhoun, 1973). For example, grape downy mildew has a high relative humidity requirement for the formation of sporangia during secondary infection. It is important to assess all likely environmental factors that may contribute to disease risk before building an infection model that can be applied to predict disease incidence (Magarey and Sutton, 2007).

Epidemiological research on *C. acutatum*

Wilson et al. (1990) showed that AFR incidence increased with longer periods of LWD, and that the LWD required for infection was less for mature compared to immature strawberry fruit. Optimum temperature for infection on both immature and mature fruit was 25 to 30° C; within this range, 80% of fruit became infected after 13 h of LWD. A regression model was generated to describe the infection level (predicted disease incidence Y) on both type of fruits using LWD (W) and temperature (T):

$$\ln (Y/[1-Y]) = -3.7 + 0.33 \times W - 0.069 \times W \times T + 0.005 \times W \times T^2 - 0.000093 \times W \times T^3$$

This equation was later used in developing a strawberry AFR warning system for use by Florida growers (MacKenzie and Peres, 2012).

Diseases caused by *Colletotrichum* species were correlated with incidence of rain splash on citrus and mango (Yang et al., 1990). Field experiments on strawberry showed convincingly that spatial dissemination of *C. actuatum* and AFR was entirely reliant on splash dispersal (Madden et al., 1993). This finding regarding splash dispersal was confirmed under controlled conditions, using a rain simulator (Madden et al., 1996).

Florida Strawberry Advisory System

In the eastern half of the U.S., AFR on strawberry is generally controlled by calendar-based fungicide applications from the start of bloom until harvest. In Florida, where the weather is highly suitable for the disease, most growers apply fungicides on a weekly schedule (MacKenzie and Peres, 2012). This practice can be expensive when considering fungicides, labor, machinery, and other associated costs. Two other problems associated with intensive fungicide use are the risk of development of pathogen resistance to the fungicides and potential health and environmental damage due to fungicide contamination (Peres et al., 2010). To mitigate these problems, a web-based disease-warning system to predict AFR epidemics on strawberries, called the 'Strawberry Advisory System' (SAS), was developed for use in northern Florida (Pavan et al., 2011; Pavan and Fraisse, 2009). It uses hourly inputs of leaf wetness duration (LWD) and temperature to predict the proportion of fruit (INF) that would become infected from AFR and gray mold (*Botrytis cinerea*) if fungicides were not applied. Florida strawberry growers can find fungicide spray guidance online (http://agroclimate.org/tools/strawberry/) by clicking their location on a map (MacKenzie and Peres, 2012). The AFR component of the SAS warning system was based on the regression model generated by experiments under controlled conditions at Ohio State University (Wilson et al. 1990), customized to northern Florida conditions by mean of field trials across multiple sites and years. SAS advises application of the fungicide captan when INF for AFR exceeds 0.15, and application of pyraclostrobin when INF exceeds 0.5. In Florida, when captan and pyraclostrobin were applied using SAS-based timing before symptoms first appeared in the field, disease control efficacy was equivalent to that achieved by weekly fungicide applications, but with an approximately 50% reduction in fungicide sprays compared to the weekly program (MacKenzie and Peres, 2012).

Validation of SAS in Iowa

Before recommending the Florida SAS t0 strawberry growers in Iowa, it is essential to validate the system under Iowa conditions (Batzer et al., 2008; Duttweiler et al., 2008; Margarey et al., 2001). This validation step is important because Iowa production conditions have fundamental differences from those in Florida. For example, Florida produces strawberries in the fall and winter, whereas harvesting in Iowa occurs during late spring and summer; the climates during these production periods differ dramatically from each other. Furthermore, strawberry production systems are dramatically different; Florida growers rely on the so-called plasticulture system, in which the period from transplanting to harvest spans a period of about 6 months, whereas Iowa's growers used primarily a perennial matted row system, supplemented by smaller plantings of day-neutral and everbearing systems. Each of these systems also utilizes different strawberry cultivars, which differ in AFR resistance among other characteristics. These profound differences between Florida and Iowa production systems explain why the SAS, which was developed for use in Florida, must undergo further field testing in Iowa before it can be recommended for grower use.

LWD, an estimate of the amount of time per day that free water is present on the leaf surface, strongly influences the risk of infection by many phytopathogens. LWD can be impacted by weather conditions (rainfall, dew, mist, wind speed), irrigation, the type of crop, its developmental stage, and the position, angle, and geometry of individual leaves (Gleason et al., 2008; Magarey and Sutton, 2007; Sutton et al., 1984). When using LWD as an input, therefore, it is advisable to perform validation trials over multiple sites and years (Batzer et al,, 2008). In Florida, LWD is monitored by sensors installed in four Florida Automated Weather Network (FAWN) stations located in the strawberry production region, so that strawberry growers do not

need to measure the weather themselves (Pavan et al., 2009). In order to make disease-warning systems easier for growers to use, LWD can be also estimated site-specifically and forecasted. These technologies have been tested for the Florida SAS system as well as for the Melcast and TOM-CAST disease-warning systems (Kim et al., 2006).

Using case studies in higher education

Case studies are educational tools that help students to engage with learning opportunities by providing scenarios that place students in the role of decision-makers in simulated real-world situations. An effective case study in plant health management, for example, keeps class discussion grounded in circumstances that actual growers must face. Case studies have been used most extensively in medicine, law, business and other professional fields (Carlson and Schodt, 1995; Richards et al., 1995).

Case studies vary in length and detail, depending on the case itself and the instructor's goals. Developers of case studies can shape the story from their own professional experiences, or from current events or historical sources. Certain factors make a case study compelling for students: a real and engaging story, a thought-provoking issue, elements of conflict, absence of an obvious or clear-cut right answer, and capturing protagonists in moments that require making significant decisions (Davis, 1993).

Case studies are a form of active learning. By using case studies, instructors can help students to discover and construct knowledge instead of just transferring their knowledge to the learner. The most important role of the instructor during a case study discussion is as a group facilitator. Students learn best when they synthesize knowledge and skills learned from a variety

of experiences and integrate their education and experience with opportunities to apply what they have learned (Tomey, 2003). Case studies in plant disease management, such as topics on cucurbit bacterial wilt and hosta takeover, had been successfully used by relevant classes (Saalau Rojas et al., 2014; Edmunds et al., 2003).

Dissertation Objectives

Colletotrichum acutatum sensu lato is a plant pathogen of key economic and scientific importance. Management of anthracnose fruit rot (AFR) caused by *C. acutatum sensu lato* on strawberry requires a reliable detection method to intercept the pathogen(s), a better understanding of the taxonomic diversity and population structure, and environmentally and ecologically rational strategies to manage the disease in the field. Consequently, my research encompassed the following two research objectives:

1. Develop a loop-mediated isotheral amplification (LAMP) assay for detecting the strawberry pathogen *C. acutatum sensu lato* in greenhouse- and field-grown plants to provide a more reliable, convenient and cost-effective tool for the strawberry industry.
2. Validate a Florida-based AFR warning system under Iowa production conditions.

A non-research objective was to develop an educational tool in the form of a case study for implementation in undergraduate education across multiple disciplines. The objectives of this case study were to help students become familiar with the “disease triangle” concept and learn how a disease-warning system uses basic principles of plant pathology to help growers minimize their use of fungicides.

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CHAPTER 2. DETECTION OF *COLLETOTRICHUM ACUTATUM SENSU LATO* ON STRAWBERRY BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

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Abstract

Colletotrichum acutatum, one of the most economically damaging pathogens of strawberry, is the primary causal agent of anthracnose fruit rot (AFR). A key challenge in managing AFR is detecting the pathogen on asymptomatic plants. To meet this need, a loop-mediated isothermal amplification (LAMP) assay was developed that incorporates two sets of primers: LITSG1 targeted on the ITS region of ribosomal DNA and Ltub2 on the β-tubulin 2 gene. In pure culture assays, Ltub2 was specific for detection of *C. acutatum*, whereas LITSG1 detected *C. acutatum* and two additional anthracnose pathogens, *C. gloeosporioides* and *C. fragariae*. LITSG1 had 10-fold lower detection threshold (20 pg of mycelial DNA) than Ltub2 (200 pg mycelial DNA) in detection of *C. acutatum* from pure culture. For detection on asymptomatic leaves, two protocols for dislodging *C. acutatum* for DNA extraction were compared: 1) the sonicate-agitate (SA) method and 2) the freeze-incubate-sonicate-agitate (FISA) method, which initially freezes tissues

followed by two days of incubation under 26 °C in darkness, then sonication and agitation. Both methods were used for greenhouse-grown plant leaves that had been spray inoculated with serial dilutions ranging from 1.5×10^{6} conidia ml^{-1} to 1.5 conidia ml^{-1}. The FISA method produced more repeatable results than the SA method. For the FISA method, detection limits (expressed as initial inoculum concentrations) using LITSG1 and Ltub2 were 1.5×10^{1} conidia ml^{-1} and 1.5×10^{2} conidia ml^{-1}, respectively. For composite samples comprised of inoculated (1.5×10^{6} conidia ml^{-1}) and non-inoculated leaves of greenhouse-grown strawberry, the two sets of LAMP primers were compared using the SA method. Primer set LITSG1 consistently detected the pathogen from a single inoculated leaf in bulk samples of 50 or fewer pathogen-free leaves whereas Ltub2 consistently detected one inoculated leaf in 20 or fewer pathogen-free leaves. Using primer set LITSG1, FISA was more sensitive than SA for detecting *C. acutatum* on leaves of field-grown plants from Florida. In an Iowa field trial using the FISA method, both primer sets detected *C. acutatum* in samples of asymptomatic leaves 6 days before fruit symptoms appeared. The results indicate that the LAMP assay has potential to provide a simplified method for detection of *C. acutatum* on asymptomatic strawberry plants.

Introduction

Colletotrichum species cause anthracnose of strawberry as well as other diseases on a wide range of hosts (Cannon et al., 2012). Three pathogens, *Colletotrichum acutatum* J.H. Simmonds, *C. gloeosporioides* (Penz.) Penz. & Sacc., and *C. fragariae* Brooks, cause sunken, brown necrotic lesions on several strawberry tissues (Howard et al., 1992; Smith 2008; Xie et al., 2010). Of the three pathogens, *C. acutatum* is the most prevalent, causing severe outbreaks of anthracnose fruit rot (AFR) as well as lesions on petioles, flowers and roots of strawberry (Howard et al., 1992; Peres et al., 2005). In the absence of symptoms, *C. acutatum* can produce

secondary conidia on leaf surfaces, which may ultimately serve as a source of primary infections on fruit (Freeman et al., 2001; Leandro et al., 2001). After colonization of host vegetative tissues in plant nurseries or production fields, the pathogen remains quiescent for weeks to months until the onset of fruit rot epidemics. Disease management is challenging when weather conditions favor disease development and production of conidia on fruit lesions (Pavan et al., 2011). Long-distance spread is mostly through transplants (Freeman et al., 2001a). Therefore, reliable and convenient detection of *C. acutatum* is needed in order to alert growers before symptoms are observed.

The taxonomic status of *C. acutatum* is in flux. Freeman et al. (2001b) used internal transcribed spacer (ITS) sequences of ribosomal DNA to place *C. acutatum* into four subgroups. Guerber et al. (2003) used phylogenetic analysis and restriction fragment length polymorphism (RFLP) of several gene introns to delineate seven clades in *C. acutatum.* Subsequently, eight worldwide subgroups were distinguished, based on 5.8S-ITS sequence information, which displayed a certain degree of correlation with host origin and geographical distribution (Sreenivasaprasad and Talhinhas, 2005). Within geographic regions, *C. acutatum* from strawberry was divided into two or more subgroups based on 5.8S-ITS sequences (Denoyes-Rothan et al., 2003; Martinez-Culebras et al., 2003; Van Hemelrijck et al., 2010). In Florida, *C. acutatum* isolates also showed some degree of host specialization and were highly pathogenic on their original hosts (MacKenzie et al., 2009). A recent study split the original *C. acutatum* complex into 30 species (Damm et al., 2012). For the present paper, however, we will consider these new taxa as *C. acutatum sensu lato* pending publication of additional research on their biology and ecology.

Several molecular detection methods for *C. acutatum* have been developed (Debode et al., 2009; Martinez-Culebras et al., 2003; Pérez-Hernández et al., 2008; Sreenivasaprasad et al. 1996). Although polymerase chain reaction (PCR) is a valuable tool to investigate the etiology of the disease (Parikka and Lemmetty 2004), significant equipment cost and technical training requirements preclude its use for routine screening of plant tissues outside of laboratory settings. Real-time PCR provides dependable detection (Debode et al., 2010), but its use by the strawberry industry is limited because the instruments are even more expensive than for conventional PCR and the methods are relatively complex.

Compared to existing molecular methods, loop-mediated isothermal amplification (LAMP) has proven to be relatively simple to conduct, cost-effective, sensitive, and specific in the case of other plant pathogens, including *Fusarium graminearum*, *Botrytis cinerea*, *Phytophthora ramorum*, and *Ralstonia solanacearum* (Kubota et al., 2008; Niessen and Vogel, 2010; Tomlinson et al., 2007; Tomlinson et al. 2010a, 2010b). The method uses six primers: four primers that recognize six regions on the target DNA and two loop primers located at the loop region of the target DNA that can accelerate the amplification (Nagamine et al., 2002). Target DNA amplification can be detected visually by means of turbidity resulting from the amplification byproduct magnesium pyrophosphate or fluorescent dye binding to double-stranded DNA (Mori et al., 2001; Tomita et al., 2008; Tomlinson et al., 2007).

The objective of this research was to develop a LAMP assay for detecting *C. acutatum sensu lato* in greenhouse- and field-grown plants to provide a more reliable, convenient and cost-effective tool for the strawberry industry.

Materials and Methods

Pathogen isolates and culture

Thirty-seven *C. acutatum sensu lato* isolates from strawberry, 46 isolates of *C. acutatum sensu lato* from other hosts, and 27 isolates of other fungi isolated from strawberry (Table 1) were maintained in 15% glycerol at -80°C. Isolates were transferred to potato dextrose agar (PDA) and incubated for 5 to 10 days at 26°C in darkness.

DNA extraction from pure cultures

Genomic DNA samples used for evaluating primer specificity were prepared from approximately 0.5 g of mycelium suspended in 40 µl of PrepMan Ultra sample preparation reagent (Life Technologies, Carlsbad, CA). The mixture was incubated at 56°C for 30 min and then at 100°C for 10 min in a thermocycler (MJ Research PTC-100TM, GMI, Inc., Ramsey, MS), and centrifuged at 13,000 rpm for 1 min. Suspensions were either used immediately as LAMP templates or stored at -20°C for later use.

LAMP primer design

The nuclear rDNA operon spanning the ITS1, 5.8S ribosomal RNA gene, and ITS2 for GenBank sequence EU647302 (MacKenzie et al., 2009) and the β-tubulin 2 gene Genbank sequence AJ209296 (Debode et al., 2009) that had been successfully used as target genes for other *C. acutatum* detection methods were selected for LAMP primer set design. The primer design software LAMP Designer (Premier Biosoft, Palo Alto, CA) generated primer set LITSG1

for the 495-bp fragment of the 5.8S and ITS2 region and primer set Ltub2 for the 550-bp β-tubulin 2 gene.

DNA amplification

LAMP reactions were performed using primer sets selected from the primer design software (Table 2). Each reaction contained 1 µL DNA extract and 24 µL of master mix; the reaction mixture contained 0.32 U/ µL Bst DNA polymerase (New England Biolabs, Ipswich, MA), 1 µL ThermoPol buffer, 1.4 mM each dNTP, 8 mM MgSO4 (including 2 mM in Thermopol buffer), 1 M betaine, 0.2 µM of each external primer (F3 and B3), 1.6 µM of each internal primer (FIP and BIP), and 0.8 µM of each loop primer (F-Loop and B-Loop). Strain 07.7 of *C. acutatum,* isolated from strawberry in Florida (Table 1), was used as a positive control and sterile distilled water (SDW) was used as a negative control. After reactions were incubated at 63°C for 50 min in a water bath, amplified products were visualized either by gel electrophoresis or by adding 2 µL PicoGreen double-stranded DNA (dsDNA) reagent (Invitrogen, Carlsbad, CA); in the latter case, fluorescence was observed under UV light. To prevent contamination, LAMP reactions and product detection were performed in different rooms.

A PCR method using primer pair CaInt2/ITS4 (Sreenivasaprasad et al., 1996) was used in *in vitro* and *in vivo* sensitivity tests as a comparison to LAMP. PCR was performed in a total volume of 25 µL, containing 1 µL DNA as template, 0.5 µM of each primer, 0.2 mM dNTPs, 5 µL 5× Green GoTaq reaction buffer, 0.4 mM $MgCl_2$, 2% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), and 1.25 units of GoTaq Flexi DNA polymerase (Promega Corp., Madison, WI). PCR amplification was performed in a thermocycler (MJ Research PTC-100TM) using the following program: 95°C for 90 s followed by 35 cycles of 95°C for 35 s, 55°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 1 min.

After primer set ITS1F/ITS4 (Gardes and Bruns, 1993; White et al., 1990) was used for sequencing the 5.8S-ITS region, the sequences were compared to the published type strains' sequences to obtain the ITS-barcode names (Table 1). Primer set TB5/TB6 (Talhinhas et al., 2002) was used for sequencing the β-tubulin 2 gene and finding the difference between LAMP-positive vs. LAMP-negative *C. acutatum sensu lato* strains. PCR for DNA sequencing was performed in a total volume of 30 µL, containing 1 µL DNA as template, 0.5 µM of each primer, 0.2 mM dNTPs, 7 µL 5× Green GoTaq reaction buffer, 0.4 mM MgCl2, 2% dimethylsulfoxide (DMSO; Sigma-Aldrich), and 1.25 units of GoTaq Flexi DNA polymerase (Promega Corp.). PCR amplifications were performed in a thermocycler (MJ Research PTC-100TM). Amplification conditions for the 5.8S-ITS region were: 95°C for 90 s followed by 35 cycles of 95°C for 35 s, 57°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 1 min; for β-tubulin 2: 95°C for 90 s followed by 35 cycles of 95°C for 35 s, 65°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 1 min. The PCR product was purified with QIAquick DNA Purification Kit (QIAgen, Valencia, CA) and automated sequencing was performed with a DNA Analyzer (Model 3730xl; Applied Biosystems) at the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA).

In vitro sensitivity

Strain 07.7 of *C. acutatum* was used for evaluating LAMP primer sensitivity. DNA extracted from mycelium was adjusted to 20 ng μl^{-1} using a spectrophotometer (Model ND-1000; NanoDrop Technologies, Inc., Wilmington, DE), then six 10-fold serial dilutions, ranging from 20 ng μl^{-1} to 0.2 pg μl^{-1}, were made with sterile distilled water (SDW); SDW served as a control. LAMP assays were then performed as previously described. As a comparison, serial dilutions were also amplified with PCR primers CaInt2/ ITS4 (Sreenivasaprasad et al., 1996).

Detection threshold on strawberry leaves

At Iowa State University, day-neutral strawberry (cv. Tristar) crowns were planted in 15-cm-diameter plastic pots containing a 1:2:1 mixture of peat, perlite, and soil and maintained in a greenhouse at 25 ± 10°C with a 16-h photoperiod. Plants were fertilized weekly with a 5% solution of 21-5-20 (N-P-K) (400 ppm N; Miracle Gro Excel, The Scotts Company, Marysville, OH), and flowers were removed twice weekly to promote development of leaves. Suspensions of *C. acutatum* conidia were prepared from 10-day-old cultures on PDA plates. Plates were flooded with SDW and colonies were scraped with sterile swabs to dislodge conidia. Suspensions were filtered through two layers of cheesecloth and then adjusted with SDW to the needed concentration using a hemacytometer. Ten-fold dilutions, ranging from 1.5×10^6 to 1.5 conidia ml^{-1}, were used as inoculum to determine LAMP detection thresholds on greenhouse-grown strawberry leaves. Forty-eight fully expanded trifoliate leaves were excised at the petiole base and brought to the laboratory. Six leaves were placed adaxial side up on wire mesh and then sprayed with SDW using an electronic atomizer (Series 571 air compressor; DeVilbiss Inc., Somerset, KY). Successive six-leaf samples were then spray inoculated with increasing concentrations of conidia, beginning with 1.5 conidia mL^{-1}. Inoculated leaves, supported on wire mesh, were transferred to plastic boxes (24 x 34 x 6 cm) (crispers) above 500 ml of SDW. The cut ends of the petioles were inserted through the mesh into the water to minimize desiccation (Leandro et al., 2001). The boxes were then sealed with plastic tape to maintain high relative humidity and incubated in darkness for 2 days at 25°C.

Each six-leaf sample was divided into two groups to compare protocols for dislodging *C. acutatum* before DNA extraction: the sonicate-agitate (SA) method and the freeze-incubate-sonicate-agitate (FISA) method (Pérez-Hernández et al., 2008). For each group, three leaves

were used as replicates. Leaves from the SA subsets were removed from the crispers, air-dried at room temperature for 30 min, and then placed in 50 ml of 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) in100-mL polyethylene bags. The bags were sonicated for 30 min in an ultrasonic cleaner (Model B-2200R-1; Branson Cleaning Equipment Company, Shelton, CT) and agitated manually for 1 min. Leaves in the FISA subsets were removed from crispers, air dried at room temperature for 30 minutes, frozen at -20°C for 3 h, and incubated at 25°C in darkness for 2 days. Each leaf was then immersed in 50 ml of 0.05% Tween 20 in a sealed sample bag, sonicated for 30 min, and manually agitated in the plastic bag for 1 min. Suspensions from SA and FISA methods were transferred to sterile 50-ml plastic tubes and centrifuged for 10 min at 4,000 rpm; supernatants were discarded and pellets were resuspended in 1.5 ml SDW, transferred to 1.5-ml Eppendorf tubes, and centrifuged for 15 min at 13,000 rpm. The pellets served as templates for total DNA extraction, using 40 μL of PrepMan Ultra sample preparation reagent as described previously.

Detection on inoculated leaves in bulked samples

To determine sensitivity of the LAMP assay in bulked samples of leaves, 21 fully expanded strawberry trifoliate leaves from the greenhouse-grown plants were collected, spray-inoculated with 1.5×10^6 conidia ml^{-1} as described previously, and incubated in crispers for 2 days. After incubation, leaves were removed from the crispers and air-dried at room temperature (18°C to 25°C) for about 30 min. Approximately 500 fully expanded, non-inoculated strawberry leaves were also collected. Next, a single inoculated trifoliate leaf was combined with either 1, 10, 20, 30, 40 or 50 non-inoculated trifoliate leaves in a plastic bag with 50, 100, 200, 300, 400 or 500 ml, respectively, of SDW containing 0.05% Tween 20. Each combination was replicated three times. The sample bags were sonicated for 30 min and manually agitated for 1 min (SA method).

From each sample, 50 ml of the extraction suspension was transferred to 50-ml plastic tubes and centrifuged for 10 min at 4,000 rpm; 1.5 ml of the precipitate, including the pellet, was transferred to an Eppendorf tube and centrifuged for 15 min at 13,000 rpm. The pellet was then re-suspended in SDW, centrifuged again for 15 min at 13,000 rpm, and used for total DNA extraction as previously described. Composite leaf samples were analyzed using both LAMP and PCR.

Detection on field-grown strawberry plants

Assays of field-grown plants were done at climatically distinct locations in central Iowa and northern Florida, using different cultivars, to assess robustness of the LAMP method. At the Iowa State University Horticulture Research Station near Gilbert, Iowa, crowns of cv. Tristar were planted on 18 May 2013 in double rows 0.3 m apart on nine 27.4-m-long beds on white-on-black plastic mulch with 0.5-m centers. On the evening of 3 Jul 2013, 15 L of a mixture of four *C. acutatum sensu lato* strains - 03.32, 05.226, 11.101, and NC-2 suspension (1.5×10^6 conidia ml^{-1}), prepared as previously described, was sprayed to runoff on all plants in the plot using a backpack sprayer. Two days after inoculation, one symptomless leaf per subplot and a total of 12 flowers were collected arbitrarily and then each sampled tissue was placed in an individual plastic bag. Fifty-four samples (42 leaves and 12 flowers) were tested individually. *C. acutatum* was extracted using the FISA method and DNA samples were prepared as previously described. As a comparison of sensitivity, extracted DNA samples were also analyzed by conventional PCR using primers CaInt2/ITS4.

In a commercial field near Wimauma, Florida, four cultivars were planted on 11 October 2013: Florida Radiance (resistant to *C. acutatum*), Sweet Charlie (resistant), Strawberry Festival (moderately resistant), and Camarosa (highly susceptible). Most of these plants were either root-

dip inoculated on the planting day or spray inoculated on 22 October 2013 with suspensions of *C. acutatum* ranging from 10^3 to 10^5 conidia ml^{-1}. Leaves were collected on 22 January 2014. Thirty leaves were selected arbitrarily from each cultivar and shipped to Iowa State University. Thirty leaves of the same cultivar were placed in one plastic bag and held in an air-padded envelope at ambient temperature for two days prior to DNA extraction. The trifoliate leaf samples were selected arbitrarily for testing by either the SA or FISA method, and the LAMP primer set LITSG1 was used for all samples from the Florida field.

Results

Assay specificity

Both primer sets amplified only *Colletotrichum* species, but primer set Ltub2 did not detect some *C. acutatum* isolates from key lime and orange (Table 1). The ITS primer set sequences (Table 2) aligned closely with the ITS region of eight distinct molecular groups within *C. acutatum* (Sreenivasaprasad and Talhinhas, 2005) but showed differences at the target sites when aligned with *C. gloeosporioides* and *C. fragariae* (Figure 1). However, *C. gloeosporioides* and *C. fragariae* were amplified by LITSG1. Because of cross-reaction of the ITS primers with the latter two species, the β-tubulin 2 gene, which has more variation among species than ITS, was chosen as a candidate target gene, and the 550-bp fragment AJ209296 was used for primer design (Debode et al., 2009). The positions of the primers as well as sequence differences of both primer sets and the target genes are shown in Figures 1 and 2. The Ltub2 primer set exhibited more base pair differences and gaps than LITSG1 at target sites with the sequences of *C. gloeosporioides* and *C. fragariae*, which allowed Ltub2 to detect *C. acutatum* exclusively. In

order to find out which new species the detected isolates belonged to, the 5.8S-ITS region and the β-tubulin 2 gene were sequenced and the closest sequences with numbers of identical base pairs were listed (Table 1). Besides *Epicoccum* and *Phoma* spp. isolates that were isolated from strawberry plants in Iowa, *Botrytis cinerea, Gnomonia* sp*., Phomopsis* sp., *Cladosporium* sp. and *Penicillium* sp. were also tested and exhibited no interference with the two LAMP primers sets.

Assay sensitivity

A ladder-like pattern in gel electrophoresis (Fig. 4A) or bright green fluorescence with PicoGreen added to the reaction tube under UV (366 nm) light (Figs. 4B and 5) indicated positive results for LAMP (Tomita et al., 2008; Tomlinson et al., 2007). The ITS primer set LITSG1 was more sensitive than the β-tubulin 2 primer set Ltub2; the amplification limits were 20 pg and 200 pg/reaction, respectively, when using template DNA extracted from pure cultures. The SDW templates showed no fluorescence in any validation test. As a comparison, PCR primers CaInt2/ ITS4 exhibited the same detection limit as LITSG1.

Sensitivity on greenhouse-grown strawberry leaves

Detection frequency of the LAMP assay was generally higher on leaves that had received more concentrated inoculum. The most sensitive detection occurred when using primers LITSG1 with the FISA protocol (Table 3). The detection limit for LITSG1 primers on leaves of greenhouse-grown strawberry, expressed as concentration of applied inoculum, was 1.5×10^{1} conidia ml^{-1} for the FISA extraction method and 1.5×10^{2} conidia ml^{-1} for the SA method. However, FISA detection success declined to one positive result per three replicates with 1.5×10^{4} conidia ml^{-1}. For Ltub2, the detection limit was 1.5×10^{3} conidia ml^{-1} for SA and 1.5×10^{2} conidia ml^{-1} for FISA.

Primer set LITSG1 consistently detected the pathogen in DNA extracted from a single inoculated leaf (1.5×10^6 conidia ml^{-1}) in bulked samples of 50 non-inoculated leaves. In contrast, Ltub2 detected the pathogen consistently when the DNA was extracted from a composite sample included one in 20 or fewer leaves and occasionally (one third of assays) one in 50 leaves. PCR using the specific primer pair CaInt2/ITS4 did not detect the pathogen in any of the composite samples (Table 4).

Detection of *C. acutatum* with LAMP assay in field samples

The LAMP assay using LITSG1 primers detected *C. acutatum* in 34 of 42 leaf samples and all 12 flower samples (Table 6) from the inoculated field in Iowa. Similarly, the LAMP assay using Ltub2 detected 31 of 42 leaf samples and 11 of 12 flower samples. PCR amplification using CaInt2/ITS4 primers showed a clearly visible amplification fragment of the correct size (490 bp) for 5 of 42 leaf samples and 8 of 12 flower samples (Table 6). Strawberries with sunken, brown lesions were first scouted in the field on 31 Jul 2013, 6 days after the leaves were sampled.

In assays of leaves from the inoculated field in Florida, LITSG1 primers detected *C. acutatum* in all four cultivars. Both DNA extraction methods had similar detection rates, except that for 'Florida Radiance' FISA was more sensitive than SA. The two resistant cultivars, 'Florida Radiance' and 'Sweet Charlie', had fewer positive reactions compared to the more susceptible cultivars with the SA method; of 15 samples tested per cultivar, positives were obtained for 3 'Florida Radiance' and 6 'Sweet Charlie' *vs.* 13 'Strawberry Festival' and 9 'Camarosa' samples. Using the FISA method, only 'Sweet Charlie' had a relatively low positive detection rate (6/15), whereas other cultivars recorded 10 or more positives among 15 assays.

Discussion

In this study we developed and validated the first LAMP assay for detection of the causal agent of strawberry anthracnose. The highly specific Ltub2 LAMP primer set reliably amplified *C. acutatum,* but not *C. gloeosporioides* or *C. fragariae*, in DNA extracts from pure cultures. The LITSG1 primer set was less specific, amplifying *C. acutatum, C. gloeosporioides* and *C. fragariae*, but had higher sensitivity. Both primer sets detected *C. acutatum* under greenhouse and field conditions. Detection of *Colletotrichum* species is challenging because of the complex nature of the pathogens; multiple species can cause similar symptoms on one host, and a single species can infect multiple hosts. The broader detection ability of LITSG1 may be advantageous in regions where all three species are present on strawberry, such as in the southeastern U.S. (Smith and Black, 1990), but also presents no disadvantage for detection in regions such as the Midwest U.S., where only *C. acutatum* is known to occur on strawberry.

LAMP offers potential advantages over PCR methods for screening of nursery transplants or production fields for the anthracnose pathogens during the symptomless phase of colonization, including lower equipment costs, less training required to conduct the assay, and increased convenience. Because it does not need a thermocycler or gel electrophoresis equipment, LAMP can be conducted in nurseries or on farms rather than in a laboratory (Tomlinson et al., 2007, 2010a). Since both primer sets detected *C. acutatum* in the field in the absence of symptoms, they may be valuable tools for detecting these pathogens on nursery transplants, although this potential remains to be tested. We also foresee that our LAMP assay could be modified readily to detect *Colletotrichum* pathogens on other hosts.

Although clearly defined genetic targets are essential for molecular recognition of pathogens, it is possible that the fluctuating taxonomic status of *Colletotrichum* pathogens could require a broader range of detection, while still excluding non-pathogenic species (Hyde et al., 2009; Sreenivasaprasad and Talhinhas 2005). Although recent phylogenetic research named 30 species in what had been considered *C. acutatum* (Damm et al., 2012), we have elected to consider these new taxa as *C. acutatum sensu lato*, because for most of the isolates there is more than one modern name associated with the two target genes: ITS and β-tubulin 2 (Table 1). Nevertheless, the findings of Damm et al. (2012) aided us in understanding the genetic background of the pathogen complex and in selecting candidate genes for LAMP primer development and further improvement, and the new species under the *C. acutatum* complex that were detected by the LAMP assay were also listed (Table 1). For some species the two detected genes gave different species names; Damm et al. (2012) indicated that β-tubulin 2 gene sequence can better identify the recently described species.

The choice of target gene directly affected the sensitivity and specificity of detection. The ITS gene is the most widely used target gene for DNA-based detection of fungi (Atkins et al., 2004). As a multi-copy gene, it also provides better detection sensitivity than single-copy genes (Debode et al., 2009). LAMP detection methods have been reported to have very high specificity (Notomi et al., 2000, Tomita et al., 2008). In the LAMP assay for *C. acutatum*, however, the 5.8s and ITS2 regions were not sufficiently variable to separate *C. acutatum* from the other two *Colletotrichum* pathogens of strawberry even though there was a seven-base-pair difference in three of the LAMP primers (Fig. 1). In contrast, the β-tubulin 2 gene of *C. acutatum* had more variability including some gaps; as a result, primers from this region were more species-specific (Fig. 2). Within *C. acutatum* recovered from a range of hosts, there were also one- to seven-base-

pair differences among subgroups, but only isolates from key lime and orange did not produce stable amplification. For example, the isolate Acardia (*C. costaricense*) had two base-pair differences in primer F2 and one base-pair difference at primer B-loop (Table 1 and Fig. 3). Since Loop primers function mainly to accelerate the speed of the amplification but are not critical to assay specificity, the difference at the 3' end of the F2 primer accounted for unstable amplifications. This result indicated that, when designing LAMP primers for species with subgroups, it is advisable to avoid the polymorphic area of the target gene located at the 3' end of primers.

To minimize the risk of contamination during transfers of LAMP amplification products in no-template controls which could lead to false positives (Tomita et al., 2008), in-tube detection with PicoGreen reagent instead of agarose gel electrophoresis was used in most of our LAMP assays. PicoGreen reagent can bind to DNA and fluoresce under UV light; it was added to each tube after amplification in a separate room from the reagent and sample preparation room in order to minimize the risk of contamination (Tomita et al., 2008).

In assays using the SA extraction method to detect the pathogen in bulked samples of leaves, the LITSG1 primer set had higher sensitivity and was more robust than Ltub2, suggesting that LITSG1 could be used in an initial screening assay for the presence of *Colletotrichum* spp. on bulked samples of strawberry plants. Our results also indicated that, for greenhouse-grown leaves, the FISA extraction method was generally more robust for LAMP detection regardless of the primer set. Similarly, Pérez-Hernández et al. (2008) showed that detection of FISA extracts was more sensitive than detection of SA extracts in conjunction with a nested PCR method for *C. acutatum* on symptomless strawberry leaves. Based on our results, the LITSG1 primer set with the FISA DNA extraction method has high potential to be used for on-site detection of *C.*

acutatum via LAMP assays.

In detecting *C. acutatum* in an Iowa strawberry field, the LAMP assay exhibited higher sensitivity and consistency than conventional PCR. Interestingly, inoculated strawberry flowers from this field were detected more readily than inoculated leaves by both LAMP and PCR; it is possible that inoculum levels on flowers were higher than on leaves because flower extracts stimulate conidium production by *C. acutatum* to a greater extent than leaf extracts (Leandro et al., 2003). Samples from the Florida field may have represented natural infection conditions more realistically than the Iowa field due to secondary dissemination during the 3-month time lag between inoculation and sampling. Resistant cultivars had lower levels of detection on leaves, suggesting that they had lower epiphytic populations of *C. acutatum* than susceptible cultivars.

This series of experiments from laboratory, greenhouse, and two field locations provides evidence for the potential value of LAMP as an on-site detection method. However, performance of the LAMP assay for pre-symptom detection of the pathogen in a naturally inoculated field with more heterogeneous spatial distribution of inoculated leaves, and determination of an optimal sampling method, remain to be evaluated in future studies. Although its detection limit is higher than that of real-time PCR or nested PCR, the LAMP assay has a significant practical advantage over these technologies because it can be adapted to plant-screening efforts on-site or outside of a conventional laboratory setting, making it more accessible to strawberry growers and their pest-management advisors.

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Tables:

Table 1. Isolates from different hosts and states of the U.S. Their modern names (Damm et al. 2012) are identified by ITS-barcode, β-tubulin 2-barcode, and reaction results in loop-mediated isothermal amplification (LAMP) assays.

Species	Host[a]	ITS-barcode[b]	β-tubulin 2-barcode	Isolates	Origin[c]	LAMP	
						ITS[d]	Tub2
C. acutatum	*Fragaria × ananassa*	*C. guajavae* (NR_111738) *and C. paxtonii* (NR_111742) 540/540	*C. nymphaeae* (JQ949848) 412/412	Cal-A, Goff, Mil-1, Mil-2, MS1ChanPop#1, OKU#1	CA, MO, MS, OK	+	+
		C. guajavae (NR_111738) *and C. paxtonii* (NR_111742) 540/540	NT	CA-1, NC1, NC2, NC3, NC4, NC5, NC6, NC7, NC8, 02.179, 03.32, 12.314	NC, FL	+	+
		C. fioriniae (NR111747) 540/540	*C. fioriniae* (JQ949943) 413/413	CF167, Cooley1, Cooley2, 01.93	FL, MA, MI	+	+
		NT	NT	07.7, 07.14, 08.27, 10.9, 10.10, 10.105, 11.342, 05.226, 09.109, 11.101, NC10, NC13, NC15, NC19, NC21	FL, NC	+	+
	Prunus persica	*C. fioriniae* (NR111747) 540/540	*C. fioriniae* (JQ949943) 413/413	Ca.Sc.PH8.04, Ca.Sc.PH9.04	SC	+	+
	Vaccinium corymbosum			04.81	CA	+	+
	Pyrus sp.	*C. fioriniae* (NR111747) 540/540	*C. fioriniae* (JQ949943) 412/413	PearFlow	MS	+	+
	Solanum lycopersicum			BJSTom, TBPop	MS	+	+

Table 1 (continued)

Rubus idaeus	*C. fioriniae* (NR111747) 540/540	*C. fioriniae* (JQ949943) 411/413	RBPop	MS	+	+
	NT	NT	04.80, 05.88, 05.218, 05.219, 05.195, 05.197, 04.52, 04.53, 05.148	CA, FL, GA, MI, MS	+	+
Citrus aurantifolia	*C. costaricense* (NR111731) *and C. limitticola* (NR_111733) 540/540	*C. limetticola* (JQ949844) 412/412	MLK1, MLK4, MLK7, KLA12.475, KLA3,	FL	+	+
	C. costaricense (NR111731) *and C. limitticola* (NR_111733) 540/540	NT	Hm-1, FL.KLA.1, FL.KLA.5, FL.KLA.7, FL.KLA.8	FL		
	C. scovillei (NR_111737) 539/540	*C. costaricense* (JQ949831) 409/412	KLA4.37	FL	+	+/-
Citrus sinensis	*C. scovillei* (NR_111737) 539/540	*C. costaricense* (JQ949831) 409/412	FL.PFD.14, FL.PFD.15, FL.PFD.16, Arcadia, Coca cola	FL	+	+/-
	C. scovillei (NR_111737) 539/540	NT	STL.FTP.1s, RCO.IMK.4	FL	+	+/-
	C. scovillei (NR_111737) 539/540	NT	MRN.IND.2s	FL	+	+
	NT	NT	FL.PFD.7, FL.PFD.8	FL	+	+

Table1 (continued)

	Rumohra adiantiformis	*C. scovillei* (NR_111737) 540/540	*C. tamarilli* (JQ949835) 411/412	05.115, 05.205	FL	+	+
		NT	NT	05.121, 05.129, 05.130, 05.133, 05.141, 05.144, 05.159	FL	+	+
C. gloeosporioides	*Fragaria × ananassa*	NR120143 494/494		95.68, 97.48A, 99.51, 00.182, 00.181, 00.117	FL	+	-
		HQ022353 495/495		96.14, 96-15A	FL	+	-
		NR120133 495/495		07.78	FL	+	-
C. fragariae	*Fragaria × ananassa*	KC790937 497/497		95.21, 03.30, 95.29	FL, MS	+	-
		HQ188923 498/498		00.176, 01.184, 07.76, 02.205, 04.99, 03.22, 02.181	FL	+	-
		HQ188923 497/498		02.178, 03.17	FL	+	-
Epicoccum sorghi	*Fragaria × ananassa*	FJ427067 440/440		EP2H5, EP1B1.2	IA	-	-
Phoma herbarum	*Fragaria × ananassa*	KJ188712 450/450		EP1B45.1, EP1B45.2	IA	-	-
Phoma pomorum	*Fragaria × ananassa*	FJ839845 458/458		EP2F34.1, EP2F34.2	IA	-	-

[a] Common names: *Fragaria* × *ananassa*, strawberry; *Prunus persica*, peach; *Pyrus sp.*, pear; *Rubus idaeus*, raspberry; *Solanum lycopersicum,* tomato; *Vaccinium corymbosum,* blueberry; *Vitis vinifera,* grape; *Citrus aurantifolia*, key lime; *Citrus sinensis*, orange; *Rumohra adiantiformis*, leatherleaf fern.

[b] *Colletotrichum* isolates and modern species names (Damm et al. 2012) based on comparison of 5.8S-ITS region and β-tubulin 2 sequence of the type strains, followed by Genbank number and the (identical base pair)/(compared base pair). "NT" =not tested.

[c] CA= California, FL= Florida, GA= Georgia, MA= Massachusetts, MI= Michigan, MO= Missouri, MS= Mississippi, NC= North Carolina, OK= Oklahoma, SC= South Carolina.

[d] +: positive, -: negative, +/-: weak or unstable reaction.

Table 2. Primers used for development of a LAMP assay for *Colletotrichum acutatum*.

Primer	Sequence
LITSG1F3	TAACAACGGATCTCTTGGTTC
LITSG1B3	GAGACGTTAGTTACTACGCAA
LITSG1FIP	TGCTCGCCAGAATGCTGGGCAGAATTCAGTGAATCATCG
LITSG1BIP	TCGAGCGTCATTTCAACCCTCGTCCGCCACTACCTTTAAG
LITSG1LoopF	CGCAATGTGCGTTCAAAGA
LITSG1LoopB	AAGCACCGCTTGGTTTTG
Ltub2F3	TAACCAGATTGGTGCTGC
Ltub2B3	TTCGTCAATAGGATTGCCTG
Ltub2FIP	TGACATACACGCCATTGCTGTATCTCGTACTGACCTTGGT
Ltub2BIP	GGACCCAGCAGCTAATCATACCTTGAAGTAGACGCTCATGC
Ltub2LoopF	GTGCTCGCCAGAGATGTT
Ltub2LoopB	ATAGGTACAACGGCACTTCC

Table 3. Comparison of LAMP primer sets LITSG1 and Ltub2 in detection of *Colletotrichum acutatum* on three inoculated strawberry leaves that were incubated for 2 days and then either 1) sonicated and agitated (SA method) or 2) frozen, incubated, sonicated, and agitated (FISA method).

Inoculum concentration[a]	SA				FISA			
	LITSG1		Ltub2		LITSG1		Ltub2	
	1[b]	2	1	2	1	2	1	2
0	0[c]	0	0	0	0	0	0	0
1.5×10^{6}	3	1	3	1	3	1	3	1
1.5×10^{5}	3	0	3	0	3	1	2	0
1.5×10^{4}	3	0	1	0	1	1	1	0
1.5×10^{3}	3	1	1	0	1	3	2	2
1.5×10^{2}	1	1	0	0	1	3	1	0
1.5×10^{1}	0	0	0	0	0	1	0	0
1.5×10^{0}	0	0	0	0	0	0	0	0

[a] Concentrations (conidia ml^{-1}) that were spray-applied on the leaves before the assays were conducted.

[b] Experiments were repeated twice; numbers indicate the two runs of the experiment.

[c] Number of leaves with positive results of assays conducted from 3 leaves total.

Table 4. Frequency of detection of *Colletotrichum acutatum* by conventional PCR and LAMP primer set LITSG1 and Ltub2 in bulked samples of inoculated and non-inoculated greenhouse-grown strawberry leaves.

# of inoculated leaves[a]	# of non-inoculated leaves	LAMP		PCR
		LITSG1	Ltub2	CaInt2/ ITS4
0	1	0[b]	0	0
1	1	3	3	0
1	10	3	2	0
1	20	3	3	0
1	30	3	0	0
1	40	3	0	0
1	50	3	1	0

[a] Number of inoculated leaves combined with clean leaves. Inoculum concentration used to spray leaves was 1.5×10^6 conidia ml^{-1}.

[b] Number of positive reactions per 3 replicate assays.

Table 5. Positive reactions for detection of *Colletotrichum acutatum* by conventional PCR (CaInt2/ ITS4 on ITS) and LAMP primer set LITSG1 and Ltub2 on individual strawberry leaves from artificially inoculated field plants (cv. Tristar) in Iowa.

Field samples (# of samples)	LAMP		PCR
	LITSG1	Ltub2	CaInt2/ ITS4
Leaf (42)	34	31	5
Flower (12)	12	11	8

Table 6. Number of strawberry leaf samples (out of 15), from artificially inoculated field plants in Florida, that tested positive for *Colletotrichum acutatum* using LAMP primer set LITSG1. .

LAMP test results	'Florida Radiance'		'Sweet Charlie'		'Strawberry Festival'		'Camarosa'	
	SA	FISA	SA	FISA	SA	FISA	SA	FISA
Positive	2	11	4	4	5	10	9	8
Weak reaction	1	1	2	2	8	1	0	4
Negative	12	3	9	9	2	4	6	3

Figures

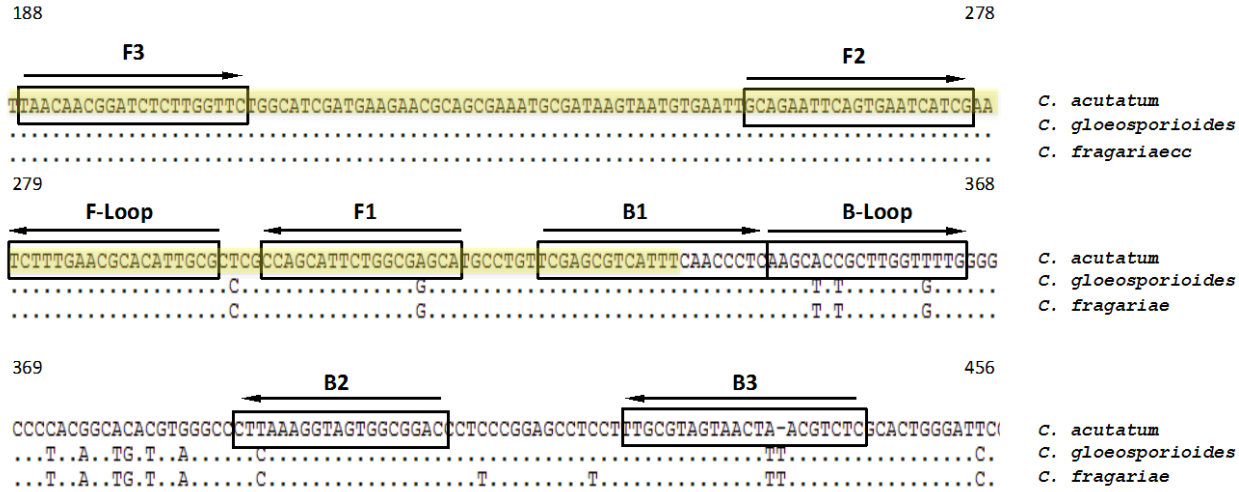


Figure 1. Positioning and orientation of loop-mediated isothermal amplification (LAMP) primer set LITSG1 aligned with the nucleotide sequences of the ITS genes of *Colletotrichum acutatum*, *C. gloeosporioides* and *C. fragariae*. Only polymorphic nucleotides are shown; (.) indicates an identical nucleotide, (−) indicates a gap in the sequence. Sequence features: 189 to 339: 5.8S ribosomal RNA (shaded) and 340 to 456: internal transcribed spacer 2.

Figure 2. Positioning and orientation of LAMP primer set Ltub2 aligned with the nucleotide sequence of the β-tubulin 2 genes of *Colletotrichum acutatum*, *C. gloeosporioides* and *C. fragariae*. Only polymorphic nucleotides are shown; (.) indicates an identical nucleotide, (−) indicates a gap in the sequence. Sequence features: exon: 1 to 27, 98 to 139 and 200 to 253 (shaded); intron: 28 to 97, 140 to 199, and 254 to 301.

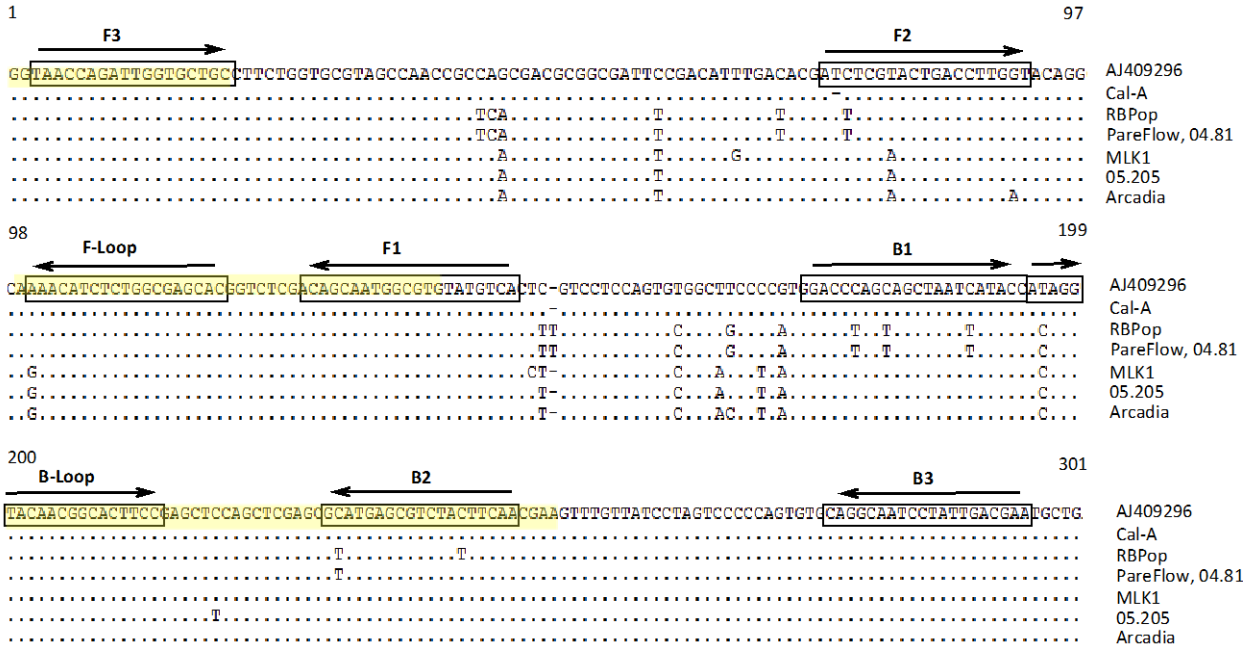
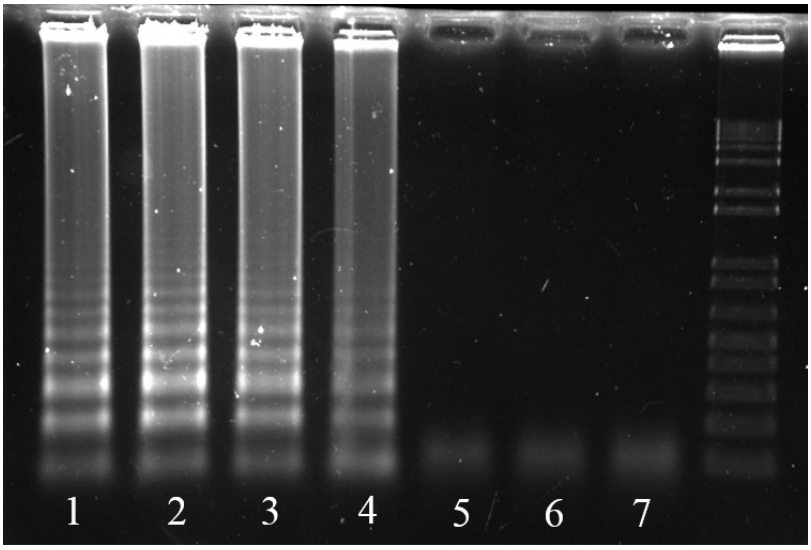


Figure 3. Positioning and orientation of LAMP primer set Ltub2 aligned with the nucleotide sequence of the β-tubulin 2 genes of representative isolates of modern species in *Colletotrichum acutatum* complex and AJ409296 sequence that used for primer design. Only polymorphic nucleotides are shown; (.) indicates an identical nucleotide, (−) indicates a gap in the sequence. Sequence features: exon: 1 to 27, 98 to 139 and 200 to 253(shaded); intron: 28 to 97, 140 to 199, and 254 to 301.

A)



B)

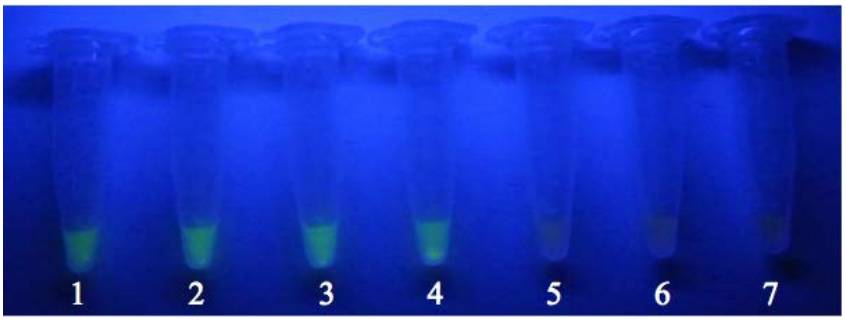


Figure 4. Sensitivity of LAMP primer set LITSG1 following incubation at 65 °C for 50 min. A serial dilution of *Colletotrichum acutatum* genomic DNA was used as template. 1= 20 ng/reaction (rxn); 2 = 2 ng/rxn; 3 = 0.2 ng/rxn; 4 = 20 pg/rxn; 5 = 2 pg/rxn; 6 = 0.2 pg/rxn; 7 = negative control (water).

A. Agarose gel electrophoresis of LAMP reactions M = 1 kb plus DNA marker.

B. PicoGreen fluorescence of LAMP reactions under UV365 nm light.

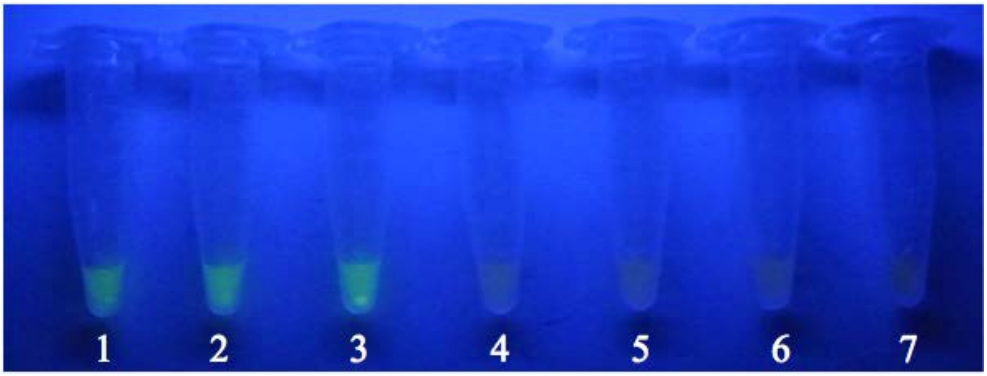


Figure 5. Sensitivity (based on PicoGreen fluorescence of LAMP reactions under UV365 nm light) of LAMP primer set Ltub2 at 65 °C for 50 min. A serial dilution of *Colletotrichum acutatum* genomic DNA was used as template. 1= 20 ng/reaction (rxn); 2 = 2 ng/rxn; 3 = 0.2 ng/rxn; 4 = 20 pg/rxn; 5 = 2 pg/rxn; 6 = 0.2 pg/rxn; 7 = negative control (water).

CHAPTER 3. VALIDATION OF A WARNING SYSTEM FOR STRAWBERRY ANTHRACNOSE FRUIT ROT IN IOWA

Will be submitted to *HortTechnology*

Abstract

Field experiments were conducted in Iowa during 2012, 2013, and 2014 to validate a disease-warning system that was previously developed and validated in Florida for strawberry anthracnose fruit rot (AFR) caused by *Colletotrichum acutatum sensu lato*. Five treatments were evaluated: two spray timing methods (warning system and calendar-based), two fungicides (captan and pyraclostrobin), and one unsprayed control. The day-neutral cultivar Tristar was spray inoculated with *C. acutatum sensu lato* during bloom. In each year, the AFR warning system saved one to two sprays compared to calendar- based treatments. In general, the warning system-based treatments performed as well as calendar-based sprays in controlling incidence of AFR. The results provide evidence that the Florida warning system can be valuable for helping Midwest strawberry growers to reduce fungicide use in managing AFR.

Introduction

Strawberry anthracnose is caused by the fungal pathogens *Colletotrichum acutatum* J.H. Simmonds *sensu lato*, *C. gloeosporioides* (Penz.) Penz. & Sacc., and *C. fragariae* Brooks. The primary pathogen of anthracnose fruit rot (AFR), however, is *C. acutatum sensu lato.* Epidemics can lead to substantial yield loss even in well-managed fields (Howard et al., 1992; Smith, 1990). *C. acutatum sensu lato* was first reported on strawberry in the U.S. in 1986 (Smith, 2008). The

pathogen can establish quiescent infections on asymptomatic nursery plants and be transported in plant shipments to production fields (Leandro et al., 2001, 2003). In the Midwest, AFR symptoms (sunken brown lesions on fruit) may appear under favorable weather conditions after fruit begin to ripen (Ellis and Madden, 1993; Leandro et al., 2001, 2003b). Although AFR was once thought to be confined to the southern U.S. (Wilson et al, 1990; Madden et al., 1996), it has become a serious disease threat throughout the Midwest during the past 25 years (Ellis and Madden, 1993).

C. acutatum is disseminated by rain splash (Yang et al., 1990). Disease incidence is related to rain intensity and duration, distance from the inoculum source, and ground cover between rows (Madden et al., 1993; 1996). Optimum temperature for *C. acutatum* infection on strawberry fruits is 25 to 30 °C, and disease incidence can exceed 80% after 13 h of wetness at these temperatures (Wilson et al., 1990). Commercial management of strawberry AFR in most of the U.S. relies on protective fungicides applied on a calendar-timed basis, but effectiveness of this strategy is erratic (Turechek et al., 2006).

A disease warning-system for strawberry AFR and gray mold (*Botrytis cinerea*), named the Strawberry Advisory System (SAS), was developed to help Florida strawberry growers optimize the efficiency of fungicide spray timing while reducing the risk of epidemics and conserving labor costs (Pavan et al., 2011). The SAS utilizes a *C. acutatum* infection model, based on inputs of leaf wetness duration (LWD) and air temperature, that predicts the risk of fruit damage (Wilson et al., 1990; MacKenzie and Peres 2012). When the estimated risk reaches a threshold, a fungicide application is recommended. This warning system is now in wide use by growers in Florida, where it has been extensively validated as reliable and cost-effective in controlling strawberry AFR (MacKenzie and Peres, 2012; Pavan et al., 2011).

Central Iowa, located about 1,900 km northwest of the main strawberry-producing region in northern Florida, has a continental climate in that differs sharply from the Florida climate. Furthermore, commercial strawberries in Florida are transplanted during September and October and harvested from November to late March, using cultivars adapted to the annual plasticulture system. Weather conditions during this period are often favorable for development of AFR epidemics; as a result, Florida growers typically sprayed fungicides weekly against AFR and gray mold until the advent of the SAS program (MacKenzie and Peres, 2012). Strawberry production systems in Iowa are more varied: June-bearing cultivars in perennial matted-row culture remain predominant, but an increasing number of growers are experimenting with annual or perennial day-neutral and plasticulture systems. The vulnerability of day-neutral cultivars to midsummer AFR epidemics is a major limiting factor to expansion of this potentially profitable production system. Because climate, production systems, and cultivars in Iowa differ from those in Florida, a first step toward adapting the SAS warning system to Iowa is to validate its performance under Iowa conditions.

Captan and pyraclostrobin fungicides are both recommended by SAS, but have different thresholds for application. Captan is a widely used contact fungicide that SAS prescribes when the risk of AFR is moderate. The alternative reduced-risk pyraclostrobin fungicides have a higher application threshold when the risk of AFR is high (MacKenzie and Peres, 2012). Pyraclostrobin, a quinone outside inhibitor (QoI) fungicide, provides more effective control than captan under prolonged wetness conditions (Peres et al, 2010; Turechek et al., 2006). The objective of this study was to validate the Florida-based SAS anthracnose warning system using two fungicides under Iowa production conditions.

Materials and Methods

Planting site, materials, and maintenance

Annual plantings of the day-neutral strawberry cultivar Tristar (Indiana Berry & Plant Co., Plymouth, IN) were established in mid- to late May at the Iowa State University Horticulture Research Station near Gilbert, Iowa from 2012 to 2014. Transplants were established in 27.4-m-long raised beds with white-on-black plastic mulch with 5-m row centers and drip irrigation in staggered double rows with 0.3-m spacing between plants. Within each mulched strip, planted subplots were alternated with non-planted strips of the same length (3 m). Each subplot contained 20 plants. Experimental design was a randomized complete block with five treatments and four replications per treatment. Treatment rows were alternated with unsprayed guard rows (cv. Tristar). For weed control, a 0.15-m-deep layer of chopped cornstalk mulch was placed between the raised beds after planting. Before plastic mulch placement, 12.33 kg/ha of urea were applied; when the plants began bearing fruit, the plot was fertigated weekly with 20-10-20 Peat-Lite plus urea (0.34 kg/ha and 1.19 kg/ha, respectively) (Petersen Products Co., Fredonia, WI). Flowers and runners were removed twice weekly until mid-July. After fruiting onset, tarnished plant bugs were controlled with two sprays of Danitol 2.4 EC (0.22 kg a.i./ha) and one spray of Assail 70 WP (200 L/ ha); insecticides were applied when incidence of tarnished plant bug damage on harvested fruit was >5%.

Inoculum preparation and inoculation

A mixture of equal proportions of four *C. acutatum sensu lato* strains - 03.32, 05.226, 11.101, and NC2 - was used for inoculations. Suspensions of *C. acutatum* conidia were prepared from

approximately 300 plates of 10-day-old cultures on potato dextrose agar (PDA) incubated at 26°C in darkness. Plates were flooded with about 10 mL sterile distilled water (SDW) and colonies were scraped with sterile swabs to dislodge conidia. Suspensions were filtered through two layers of cheesecloth, and with the aid of a hemacytometer adjusted to final concentrations ranging from 1.5×10^4 to 1.5×10^6 conidia ml^{-1}; the lower concentration was used when weather conditions at the time of inoculation were highly favorable to inoculum survival and dissemination, whereas the higher concentration was used under less favorable weather conditions. Inoculations were performed at dusk in late July; during each inoculation, a total of 15 L of *C. acutatum* suspension was sprayed to runoff on all plants in the plot using a 15-L hand-pressurized backpack sprayer (Model 452, Solo, Newport News, VA). Overhead irrigation was applied for 30 min before and after the inoculation to promote pathogen colonization and infection.

Treatment application

Five treatments were evaluated that included two spray timing methods (warning system and calendar), two fungicides (Captan and reduced-risk fungicide Cabrio), and one unsprayed control. Calendar-based sprays were applied every 10 days, beginning when removal of flowers ceased in 2012 and after inoculation in 2013 and 2014. For disease−warning system based sprays, two thresholds were set: a moderate risk threshold, for which Captan 80 WDG was applied at 1.65 kg a.i./ha (3.75 lb/A) and a high-risk threshold, for which Cabrio 20EG was applied at 0.196 kg a.i./ha (14 oz/A). The predicted level of disease risk (INF) was calculated with the following equation:

$\ln(INF/[1 - INF]) = -3.70 + 0.33W - 0.069WT + 0.0050WT^2 - 0.93 \times 10^{-4} WT^3$ where W= leaf wetness duration and T=temperature during wet period). Moderate risk was assumed to

occur when INF was >0.15, and high risk when INF was >0.5 (MacKenzie and Peres, 2012).

Data collection and statistical analysis

In 2012 fungicide applications started from July 1 before inoculation; the calendar-based fungicide spray was applied on July 2 and 12, and the warning system treatments were sprayed on July 6 before inoculation at July 18. In 2013 and 2014, fungicides applications started after inoculation: the warning system was initiated when the first warning was received after inoculation and the calendar spray was started at the same time.

Leaf wetness duration (LWD) was measured using two printed-circuit sensors (model 237; Campbell Scientific, Logan, UT) coated with off-white latex paint. Sensors were mounted on the end of a section of PVC pipe at an inclination of 45° to horizontal. Free water on the leaf wetness sensors was detected by measuring the electrical impedance of the grid using a datalogger (model CR10; Campbell Scientific, Logan, UT). Temperature was measured by two temperature probes (Model 107, Campbell Scientific) that were placed inside the PVC pipes. Hourly average LWD and temperature were downloaded twice weekly and used to calculate INF from July 1 until the end of harvest in each year.

Mature berries were harvested three times at weekly intervals when weather and pesticide pre-harvest intervals allowed, from late July to September. For each subplot, weight and number of marketable fruit, culls (damage by other reason such as rot, sunburn, animal or insects), and fruit displaying anthracnose fruit rot (AFR) symptoms were recorded. Disease incidence, marketable yield, AFR yield and cull yield were compared to evaluate the effect of treatments.

Disease incidence data were pooled to construct an overall incidence value for each replicate of each treatment. The angular transformation of incidence was calculated to obtain a response variable with an approximately constant variance. The MIXED procedure of SAS (SAS Institute,

Inc. Cary, NC) was used to determine the effect of treatment on disease, based on fitting a linear mixed model to the angular-transformed values. Treatment was considered a fixed effect and block a random effect. Significant differences of the estimated least-squares means were determined based on the least significant difference (LSD; P = 0.05).

Results

Weather conditions and AFR epidemics

Weather conditions were rainy and >20 °C, suitable for *C. acutatum* dissemination and infection, on the inoculation days during 2012 and 2014 (Leandro et al., 2003a). In August of 2012, prolonged dry periods with maximum daytime temperatures >35 °C inhibited AFR disease development (Figure 1A), so disease incidence for the unsprayed control treatment remained below 20% (Table 1) (Leandro et al. 2003a; Wilson et al. 1990). For 2014, weather was generally warmer and wetter (Figure 1C); warning system thresholds were reached >20 times during the harvest season, and AFR disease incidence for the control treatment reached 57.3% (Table 3). Weather conditions during 2013 were exceptionally dry; although the day of inoculation was rainy (12 h of leaf wetness duration), only 7 warnings were received thereafter, and AFR incidence for the control treatment was <4% (Table 2).

Anthracnose fruit rot incidence and yield

The warning system saved one to two sprays compared to calendar-based treatments in each year and reduced AFR incidence as effectively as calendar-based sprays. In 2012 and 2014, all fungicide treatments controlled AFR significantly ($P<0.05$) compared to the unsprayed treatment (Tables 1 and 3).

In 2012, the warning system treatments saved two fungicide sprays and did not differ significantly ($P>0.05$) from the calendar-based treatment in controlling AFR incidence; each suppressed disease incidence by about 15% compared to the unsprayed treatment (Table 1). However, marketable fruit weight comparisons revealed that the warning-system's Captan-only treatment (trt 3) performed as well as the calendar-based treatments, but that the warning-system treatment with alternated fungicides (trt 4) had lower marketable yield than other three.

In 2013, both calendar-based treatments significantly ($P<0.05$) resulted in AFR incidence that was about 57% lower than the unsprayed treatment. The warning system treatments saved one fungicide spray and were as effective as the calendar-based treatments ($P>0.05$). Low disease pressure resulted in lack of significant differences between the warning system treatments and unsprayed control, although the warning system resulted in 36% lower disease incidence (Table 2). However, the marketable weight comparisons showed that all the treatments performed the same.

In 2014, all fungicide treatments significantly ($P<0.05$) suppressed AFR compared to the unsprayed treatment, which reached 100% disease loss during some bi-weekly harvests. The warning system treatments saved one fungicide spray and were as effective as the calendar-based treatments ($P>0.05$). Cabrio treatments resulted in slightly but not significantly more control than the conventional Captan fungicide treatments. All sprayed treatments had less AFR and higher marketable weight than the unsprayed treatment ($P<0.05$).

During each year, damage on the fruit caused by other factors, such as rot, sunburn, animal or insects, did not differ among treatments.

Discussion

Despite being transported 1,900 km to a different climate, strawberry production system, and cultivar, the Florida-derived AFR warning system performed consistently well in Iowa across a wide range of weather conditions. In a day-neutral strawberry production system, warning system treatments saved one to two fungicide applications per season while achieving levels of AFR control equivalent to fungicide application on a 10-day calendar basis.

Our findings provide preliminary evidence that SAS can be adapted to Iowa growing conditions, despite many differences from Florida strawberry production. However, our results also suggest that the magnitude of fungicide-spray savings from using SAS in Iowa, and possibly in other parts of the Midwest, is likely to be less than in Florida, presumably because disease-favorable weather conditions are less prevalent in the Midwest than in Florida during the period of fruit maturation (MacKenzie and Peres, 2012). Optimizing SAS for use in the Midwest will require further validation at multiple locations using a range of production systems including perennial June-bearing and annual plasticulture as well as day-neutral systems; it is reasonable to hypothesize that SAS algorithms will need to be modified to optimize the system's performance in different Midwest production scenarios (Duttweiler et al., 2008; Gleason et al., 2008; Magarey et al., 2001). In addition, it is essential to quantify the economic impact of using SAS under these circumstances as well as grower receptivity toward this change in anthracnose management tactics (Sherman and Gent, 2014). Nevertheless, our results provide a starting point for these additional steps toward integrating SAS into grower practice in the Midwest.

In order to prevent contamination by *C. acutatum* from the nursery or plant debris, fungicides were applied before inoculation during 2012. However, we later suspected that this step was not

necessary under the conditions of this experiment (annual cropping, rotating to ground that had not been cropped to strawberries in at least 3 years). In 2013 and 2014, therefore, we altered our methods by applying fungicides after inoculation, and initiated the calendar-based and warning-system treatments at the same time. In each case, the warning system worked in an equivalent manner to suppress AFR. In situations in which over-seasoning inoculum may constitute a substantial risk, however, it may be necessary to initiate fungicide spraying during the period between transplanting and the start of fruit development.

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Table 1. Treatments, anthracnose fruit rot (AFR), and yield data summary at the Iowa State University Horticultural Research Station, Gilbert, IA during 2012.

						Yield per 20 plants (g)		
Treatment	**Fungicide**	**Timing Schedule**	**Period**	**Spray #**	**AFR[a] incidence %**	**Marketable wt[b]**	**AFR wt**	**Cull wt[c]**
1	Captan 80WP	10 days	July1 to Sept 15	7	2.78 A	223.81 A	5.78 A	22.13 A
2	Captan 80WP Cabrio 20EG	10 days 10 days	July1 to July 31 Aug1 to Sept 15	7	3.08 A	216.10 A	6.38 A	18.49 A
3	Captan 80WP	Warning system	July1 to Sept 15	5	6.61 A	217.51 A	16.78 B	25.10 A
4	Captan 80WP Cabrio 20EG	Warning system; alternated fungicides	July1 to Sept 15	5	2.22 A	176.79 AB	3.99 A	22.26 A
5	None	NA		0	19.09 B	149.63 B	30.40 C	20.76 A

a. Means followed by the same letter are not significantly different within column according to Fisher's protected LSD at $P \leq 0.05$.

b. Marketable yield is the average yield of marketable fruit per 20-plant subplot.

c. Cull yield is the average weight including fruit damaged by other rots, and insect pests per 20-plant subplot

Table 2. Treatments, anthracnose fruit rot (AFR), and yield data summary at the Iowa State University Horticultural Research Station, Gilbert, IA during 2013.

						Yield per 20 plants (g)		
Treatment	**Fungicide**	**Timing Schedule**	**Period**	**Spray #**	**AFR[a] incidence %**	**Marketable wt[b]**	**AFR wt**	**Cull wt[c]**
1	Captan 80WP	10 days	July1 to Sept 15	4	1.73 A	255.58 A	4.19 A	42.18 A
2	Captan 80WP Cabrio 20EG	10 days 10 days	July1 to July 31 Aug1 to Sept 15	4	1.38 A	245.60 A	2.55 A	40.02 A
3	Captan 80WP	Warning system	July1 to Sept 15	3	2.54 AB	242.66 A	5.45 AB	41.10 A
4	Captan 80WP Cabrio 20EG	Warning system; alternated fungicides	July1 to Sept 15	3	2.08 AB	247.67 A	4.76 AB	39.82 A
5	None	NA		0	3.64 B	260.73 A	8.89 B	37.70 A

a. Means followed by the same letter are not significantly different within column according to Fisher's protected LSD at $P \leq 0.05$.

b. Marketable yield is the average yield of marketable fruit per 20-plant subplot.

c. Cull yield is the average weight including fruit damaged by other rots, and insect pests per 20-plant subplot

Table 3. Treatments, anthracnose fruit rot (AFR), and yield data summary at the Iowa State University Horticultural Research Station, Gilbert, IA during 2014.

						Yield per 20 plants (g)		
Treatment	**Fungicide**	**Timing Schedule**	**Period**	**Spray #**	**AFR[a] incidence %**	**Marketable wt[b]**	**AFR wt**	**Cull wt[c]**
1	Captan 80WP	10 days	July1 to Sept 15	6	13.55 A	170.08 A	26.01 A	15.30 A
2	Captan 80WP Cabrio 20EG	10 days 10 days	July1 to July 31 Aug1 to Sept 15	6	8.24 A	191.65 A	12.14 A	15.71 A
3	Captan 80WP	Warning system	July1 to Sept 15	5	13.59 A	157.55 A	25.50 A	15.73 A
4	Captan 80WP Cabrio 20EG	Warning system; alternated fungicides	July1 to Sept 15	5	8.33 A	170.21 A	10.21 A	12.93 A
5	None	NA		0	57.30 B	44.71 B	91.51 B	13.39 A

a. Means followed by the same letter are not significantly different within column according to Fisher's protected LSD at $P \leq 0.05$.

b. Marketable yield is the average yield of marketable fruit per 20-plant subplot.

c. Cull yield is the average weight including fruit damaged by other rots, and insect pests per 20-plant subplot

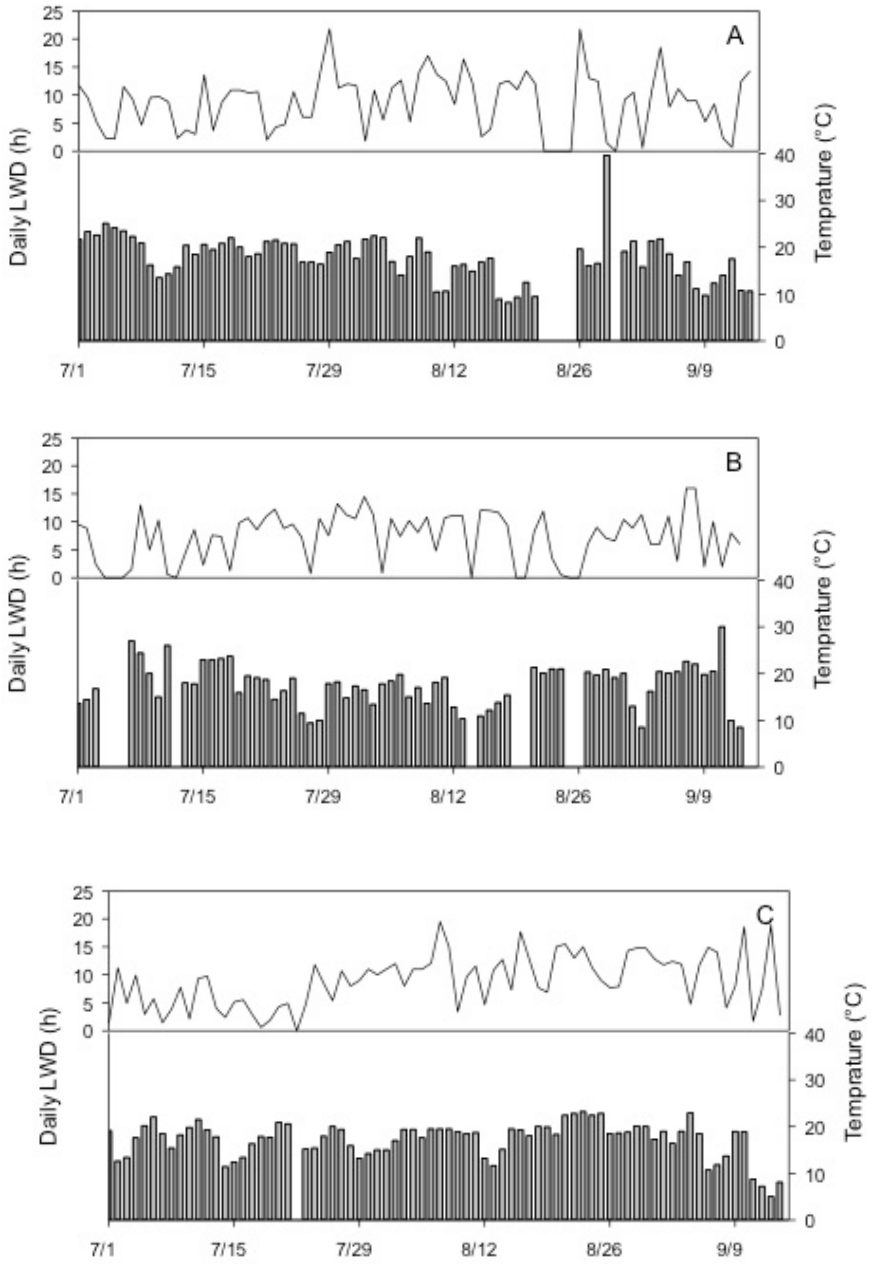

Figure 1. Daily leaf wetness duration (LWD; solid line) in hours per day, and average daily temperature during the same period (vertical bars) for 2012 (A), 2013 (B), and 2014 (C). Missing bars indicate periods during which no LWD was recorded.

CHAPTER 4. CHAPTER STRAWBERRY ANTHRACNOSE: MANAGING A HIDDEN MENACE

Accepted by *Plant Health Instructor*

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Introduction

"Strawberry anthracnose: managing a hidden menace" is a case study that highlights several basic principles of plant disease management and introduces a type of ecologically based management strategy called a disease-warning system. Students are given background on a situation in which a well-established Iowa strawberry grower, Jack O'Neil, is struggling to suppress a damaging fungal disease, anthracnose fruit rot (AFR). The fungus that causes AFR is unusually challenging to control because it can remain invisible in a strawberry field for long periods and then suddenly erupt, causing devastating fruit rot epidemics.

Jack's crop is threatened by AFR. In order to protect his livelihood, he sprays fungicides on his fields every 10 days. Now, however, many customers who pick strawberries on his farm are pressuring him to cut back on fungicide sprays. They are worried that fungicide residues on the plants and berries could endanger their health as well as the safety of the local groundwater,

which is the source of drinking water for the community. But the customers still want beautiful berries without the sunken brown spots caused by AFR. How can Jack solve this dilemma and still manage to stay in business?

Students are challenged to help Jack decide how to manage AFR with fewer fungicide sprays so that he can meet his customers' demands. As students study this case, they will learn how plants become infected and how a disease-warning system uses information about the weather to help growers manage diseases with less reliance on fungicides.

Objectives

The overall goals of this case study are to help students become familiar with the "disease triangle" concept and learn how a disease-warning system uses basic principles of plant pathology to help growers minimize their use of fungicides. Students will be challenged to help Iowa strawberry grower Jack O'Neil decide whether or not to use the warning system on his farm. From this case study, the students will:

- describe the disease triangle concept and explain how it relates to real-world disease management tactics,
- understand the life cycle of the pathogen (disease-causing organism) of AFR, a fungus named *Colletotrichum acutatum,* and how it causes anthracnose fruit rot (AFR), and
- examine the pros and cons of using a disease-warning system in a real-world situation.

Cast of characters

Jack O'Neil: A strawberry grower who owns Sunny Patch Farm in central Iowa; he grows 15 acres of strawberries. The most profitable segment of Jack's business comes from "pick-your-own" customers who visit his farm to harvest their own berries. In addition, he sells pre-picked

fruit at a local farmer's market. In the last 2 years, many of his customers, responding to scary news reports about serious health and environmental risks of pesticides, have asked about – and even insisted – that Jack sharply reduce the number of pesticide sprays he applies. Since most of Jack's pesticide sprays consist of fungicides, his customers' concern has zeroed in on fungicides.

Anna Nasser: Jack's neighbor, who told Jack about her own family's concerns – as well as those of 30 additional families who are Jack's customers – regarding pesticide use in his pick-your-own strawberry fields. Anna has even threatened to organize a customer boycott of Sunny Patch Farm if Jack does not respond quickly to their concerns.

Dr. Nancy Muller: An extension plant pathologist at Iowa State University who researches strawberry diseases and advises growers on disease management of fruit crops.

Classroom Management

Case Summary

This case is intended to help undergraduate students grasp the basic principles of the disease triangle, how weather conditions affect plant disease risk, and how growers can use disease-warning systems to manage threatening diseases in a more efficient and environmentally friendly way. Students will learn these concepts while gaining a basic understanding of anthracnose fruit rot (AFR) of strawberry, a difficult-to-manage threat because the pathogen can remain hidden for long periods and then suddenly break out in devastating epidemics. In addition, the case exposes students to the challenges of disease management in a situation where customers and their families visit the farm, handle the berries with their bare hands, and often eat the fruit without washing them. The farmer in the case, Jack O'Neil, must keep AFR under control while somehow responding to his customers' demands to use less fungicide. When extension plant pathologist Nancy Muller suggests using a disease-warning system to meet his needs, Jack needs to get up to speed quickly on this new approach before trying it. Students will place themselves in the role of Jack, the owner of Sunny Patch Farm, and propose their own management plan based on the information provided. Students will gain a deeper understanding of real-world disease management in a high-value fruit crop and will analyze information to help Jack make decisions.

How to use this case

This case is designed for use in a single 50- to 90-minute class. We suggest that Part I, which concerns basic information explaining why AFR poses such a threat to Jack's strawberries, be

assigned reading that is completed before the class period. The background information provided, which briefly describes how AFR attacks strawberries, should also be read before the class session.

During the first part of the class, we suggest that the instructor share the Part I information and present a short summary of AFR using a mind map (Willis & Miertschin, 2006). Each student can write down his/her own answers on the mind map before and during the instructor's presentation. There are two mind maps designed for class use: one for the beginner level, which gives students more guidance, and one for the advanced level. After the review, the class can be divided into small groups (for example, 3 to 4 students each) to discuss their individual answers among themselves for a few minutes, after which a representative from each group can present the group's consensus answers for Part I.

After reviewing the disease information and discussing Part I, students will be given 5 minutes to read Part II and then discuss answers to Part II questions in their groups for 10 minutes. As with Part I, each group should come up with their answers and then share these with the entire class using the mind map. Evaluating Part II questions can also be assigned as out-of-class group homework, where students bring their group's answers to the next class period to share with the entire class.

This case study can be used for classes in plant pathology, horticulture, sustainable agriculture, integrated pest management, plant health management, and agricultural education. The case focuses on managing a tough-to-control disease with less reliance on fungicide by using a weather-based warning system. To decide whether it makes sense to use the warning system,

the students need to understand the grower's farming environment as well as the advantages and risks of the warning system.

The Case

Part I

Jack's farm

Jack O'Neil owns Sunny Patch Farm, located near Des Moines, Iowa, with 15 acres in strawberry production. Most of his income is from customers and their families who come out to Sunny Patch Farm to pick their own berries (Figure 1) and buy Jack's homemade jams and pies. In addition, Jack sells pre-picked strawberries twice a week at the Des Moines Farmers Market.

Figure 1. Pick-your-own customers at Sunny Patch Farm

Strawberry is one of the most productive and high-value crops in the world, but it must be managed carefully to suppress diseases and insect pests that can damage the crop. Jack's main defense against strawberry diseases is spraying numerous applications of synthetic chemical fungicides on the crop.

The strawberry harvest comes in a 4-week-long period during late May and early June. He harvests the planting in years 2 to 5, and then rotates to another crop on that site for 3 years while harvesting strawberries from additional fields. This "matted row" system maintains berry production while reducing the risk of spreading diseases – including anthracnose fruit rot - from

one strawberry planting to the next. Because Jack farms in a cold climate, he covers his strawberries with 4 inches of oat straw each year in late November to avoid freeze damage to the plants during the winter months. In April, he moves some of the straw off the plants and places it between the rows, but also packs straw around the strawberry plants in order to reduce the risk of soil splashing onto the berries. The income and costs for Jack's farm are estimated and listed in Table 1.

Table 1. Sunny Patch Farm's annual income and costs (per acre).

Fruit type	Gross income	Production costs			Other costs (taxes, etc.)
		Labor (Harvest only)	Nursery plants	Management	
U-pick	$10,000	$4,000	$1000	$1,000	$110
Pre-Picked	$12,000	$6,000	$1000	$1,200	$110

Figure 2. Anthracnose fruit rot on ripe strawberry

Anthracnose fruit rot: an insidious enemy.

The disease that scares Jack the most is anthracnose fruit rot (AFR). The fungus that causes AFR, *Colletotrichum acutatum,* is notorious for its sneaky behavior. Unlike most fungi that

cause crop diseases, this one can hang around on leaves and stems for a long time without showing any symptoms (symptoms = visible effects of a disease on plants). But when the weather becomes warm and rainy, *C. acutatum* suddenly can attack ripening fruit, causing sunken, brown spots that make the fruit unfit for sale (Figure 2) (Louws et al., n.d.). An AFR epidemic can spell disaster for a commercial farm like Jack's: more than half of the fruit can be ruined.

Twenty years ago, shortly after Jack had started his strawberry business, he was hit by an AFR epidemic. Walking his fields one day after a thunderstorm, just as the harvest period was beginning, he noticed a few fruit with sunken, brown spots (Figure 2). The very next day, the damaged fruit were five times more numerous. Despite immediately starting to fight back with fungicide sprays, he ended up losing more than 70% of his crop to AFR. He was even forced to suspend pick-your-own activities and farmers-market sales that year, disappointing scores of his customers. He worried they might not return to his farm next year or just buy non-local strawberries in the grocery store.

In addition to taking a serious financial hit, Jack was bewildered by the suddenness of the AFR outbreak and how badly he had failed to stop it. So he sought advice from Dr. Nancy Muller, a plant pathologist at Iowa State University and an expert on strawberry diseases. Nancy told

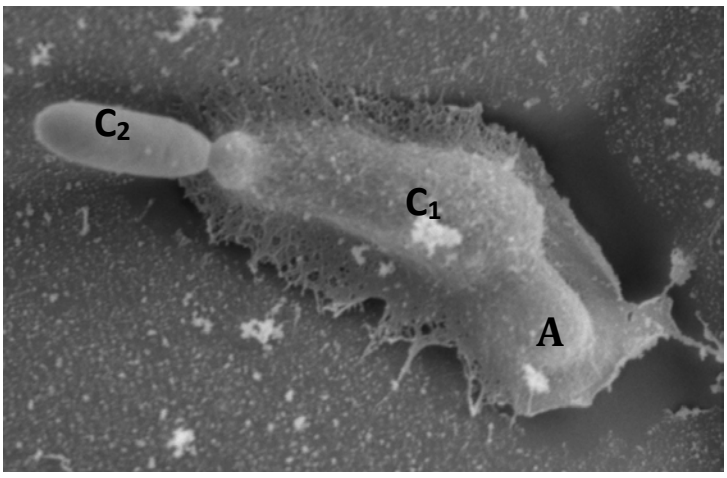

Figure 3. Conidium (C_1) and appressorium (A) attached to the leaf surface produce a new conidium (C_2).

Jack about AFR's ability to hide in plain sight: its microscopic spores, called conidia, can spread rapidly across a field during warm, rainy weather, establishing invisible infections on plants. Once fruit began to ripen, further rainy weather could cause innumerable fruit infections and a crop failure like the one Jack had experienced.

The fungus that causes AFR, *Colletotrichum acutatum,* is a hitchhiker. It can stick to nursery plants with its own natural glue and a special survival structure called an appressorium (Figure 3, A), which is too small to see with the naked eye, and then move hundreds of miles in a shipment of young plants to Jack's farm. Once the plants start growing, warmth and rainfall wake up the fungus and it uses its asexual spores, called conidia (Figure 3, C), which move in raindrops, to spread to other plants. Twenty years ago, when Jack noticed the fruit rot symptoms it was already game over— too late for him to control the disease. In the aftermath of that disaster, Nancy suggested that Jack spray fungicides on his fields every 10 days during and after the flowering period to keep AFR under control.

Since then, Jack has followed Nancy's spray advice to the letter. He has never had another AFR epidemic, but sometimes wonders whether it's necessary to apply so many fungicide sprays.

Current disease management strategies at Sunny Patch Farm

Every year, Sunny Patch Farm purchases strawberry crowns (small plants with roots and leaves attached) from nurseries in other states. Jack picks strawberry varieties that grow well in Iowa and whose fruit have excellent flavor, uniformly red color, and high yields, such as Honeoye, Jewel and Kent. However, all of these varieties are susceptible to AFR, so fungicide sprays must be part of his AFR management program.

When rainfall and dew during the flowering and fruit ripening period keep plants wet for more than 12 hours at a time and average temperature ranges from 77° F (25 C) to 86° F (30 C), *C. acutatum* will spread through a strawberry field, infect leaves and fruit invisibly, and eventually cause AFR symptoms. In Iowa, these disease-favorable conditions occur in some years but not others; in other words, they are sporadic. But when disease-favorable conditions do occur, and especially if they are prolonged, AFR can become a raging epidemic unless Jack uses fungicide sprays. Because he cannot predict weather, he views each year as a potential AFR outbreak. He sees fungicide as "cheap insurance," since fungicide spraying is much less expensive for him than enduring an AFR epidemic. In a typical growing season, he applies five sprays against AFR.

PART I QUESTIONS:

1. What characteristics of the fungus *Colletotrichum acutatum* can cause anthracnose fruit rot (AFR) to appear so suddenly in a field?
2. What are the roles of conidia and appressoria in spreading the disease?
3. What are suitable weather conditions for AFR to develop?
4. What strategies is Jack currently using for AFR control (including fungicides and all the cultural practices)? How does each of these strategies reduce the threat of an AFR outbreak?
5. a. What is the net income of Sunny Patch Farm when Jack has 70% of his field for U-Pick and 30% for pre-picked strawberry?

   b. What is the net income of Jack's farm when it has 70% yield loss due to an AFR outbreak?

   *Net income= Gross income - total cost

* During an AFR epidemic, assume that Jack's labor cost is reduced by 50% due to loss of harvestable yield.

Part II

Customers push for a change

The past few years have brought Jack an increasing chorus of questions and complaints from strawberry customers about his rigorous program of fungicide spraying. Alarmed by media reports of health hazards associated with pesticides, many parents have expressed concern to Jack about the possible dangers of eating strawberries that could have pesticide residues on them. One day Jack got complaints from his neighbor and friend Anna Nasser, a long-time customer who always brings her children to his farm to pick their own strawberries.

"Jack," Anna said, "we love to bring our family out to pick strawberries, but we're worried about all that fungicide you use. I want to keep my kids safe, so I may have to start buying berries from farms that use fewer chemicals. But I'd really rather buy from Sunny Patch Farm; is there any way you can cut back on the fungicides?" Anna's concerns remind Jack that he has been having similar conversations with other customers, neighbors, and friends. Even at the farmers market, more and more customers want to know about Jack's fungicide program on the berries.

Jack already knows that fungicide use has some potential for risks to human health and the environment. But he wants to argue that not all of the fungicides pose the same risk. For example, pyraclostrobin (Cabrio), a fungicide in the strobilurin group that is designated by the U.S. Environmental Protection Agency (EPA) as 'reduced risk,' is much less damaging than Captan, an older fungicide that Jack also uses. Even though Cabrio is more effective against AFR than Captan, Jack can't just rely on Cabrio for every spray, because there is a high risk that *C. acutatum* will become resistant to it after many consecutive sprays. In fact, resistance has already started to appear. Growing customer pressure is forcing him to re-evaluate his disease management practices in order to remain in business. How can he cut down on fungicide use and still produce top-quality strawberries? Looking for answers, Jack phones Nancy Muller, the plant pathologist.

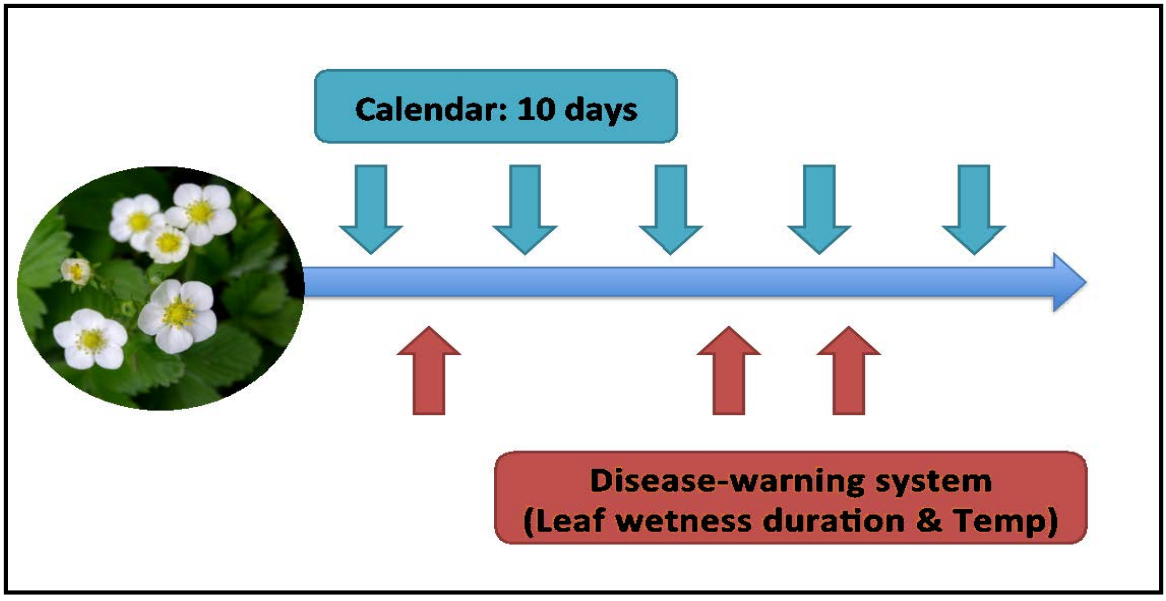

Figure 4. Example of fungicide spray timing according to two management systems: calendar-based (every 10 days) and a disease-warning system (applying only when a weather-based threshold is reached).

Disease-warning system

"Hi Nancy, is there a way I can control anthracnose fruit rot with less fungicide? My customers are on my back all the time about this."

"It's good to hear from you, Jack. There may be a way around this problem. We've been field-testing a **disease-warning system** for anthracnose fruit rot that can help you time fungicide sprays based on the weather conditions. A disease-warning system is a tool for optimizing plant disease management. It uses the information about one or more components of the disease triangle - environment, host, and pathogen – to predict the risk of a disease epidemic (Campbell and Madden, 1990). Warning systems have been used successfully on a range of vegetable and fruit crops, including tomato and cantaloupe (http://extension.udel.edu/weeklycropupdate/?p=220).

A warning system for AFR on strawberry, called the Florida Advisory System (FAS), was originally developed in Florida (MacKenzie and Peres, 2012). Growers can access the website (http://agroclimate.org/tools/Strawberry-Advisory-System/) to get fungicide spray guidance. Recently, FAS has shown promise for use in the Midwest. Based on our results, you can save an average of two fungicide sprays per season, and still keep AFR under control. Maybe you'd like to try it out?" Nancy then emails Jack some details about the FAS warning system:

- Using an equation developed from results of studies of *C. acutatum* biology (Wilson et al., 1990), the FAS warning system rates the risk of an AFR outbreak based on the number of hours per day when plants are wet (referred to as leaf wetness duration, or LWD) and the average air temperature during these wet periods. It then advises you whether or not a fungicide spray is needed.

- You can monitor the weather for the warning system using a weather station that includes sensors to track the weather conditions, a datalogger to record the weather data, and a built-in computer chip that runs the equation to convert the weather data into an AFR risk rating. The risk rating can be accessed by simply pressing a button on the datalogger. The price of the equipment ranges from $500 to $3,000, depending on quality: the higher-priced equipment is more reliable and lasts longer. Location of the weather station also affects risk prediction, because some locations on the farm stay wet for longer periods after rain or dew than other locations.
- There are pros and cons to consider in deciding whether to try out the AFR warning system:
    - On the positive side, you can show your customers that fungicide usage is reduced by 40% (by eliminating an average of 2 fungicide sprays from your usual total of 5 sprays per season). You will save some money and time by avoiding two fungicide sprays per year as well as protect the environment, yourself, and your customers. On top of that, using less fungicide may also reduce the risk of developing *C. acutatum* that is resistant to fungicides.
    - On the negative side, you may worry that the risk of an AFR outbreak is increasing by abandoning your "tried and true" practice of making 5 fungicide sprays per season and placing your trust in a new, weather-timed spray system. The calendar sprays are more predictable than the disease-warning system sprays, so you can schedule them more efficiently. Warning-system spray alerts, on the other hand, may come at less convenient times, such as when the farm is open for fruit picking or when the weather is too wet to spray fungicide. As you know, a serious outbreak of AFR could cost you far more in lost sales than what you would save by applying 2 fewer fungicide sprays

per season. In addition, tending a weather station will take some additional time and trouble during a busy growing season.

Should Jack trust his strawberry crop to the warning system and the weather monitoring equipment? Is it worth the extra time and trouble? Strawberry is such a high-profit crop that Jack really doesn't want to make any mistakes. But he doesn't want to lose customers either. What should he do?

PART II QUESTIONS:

1. Describe how the AFR warning system works.
2. Based on the disease triangle concept:
    a. Are there strategies that Jack could use to keep *C. acutatum* from getting into his fields?
    b. If the fungi were confirmed NOT to be present in the field, would it make sense to spray fungicides against AFR anyway? Why or why not?
3. Based on the information presented in the case:
    a. Would you use the warning system if you were Jack O'Neil?
    b. If yes, how can he make sure that the warning system is working in his field? What are possible problems associated with using it? What additional information would be useful to help you decide?
    c. How will using the warning system help Jack to reduce the risk of resistance development?
    d. If Jack decides not to use the warning system, how can he meet his customers' demands that he cut back on fungicide use?

Background Information

A short summary of AFR

Strawberry is a popular fruit because of its beautiful color, attractive flavor and widely adaptable nature. The United States is one of the top three strawberry- producing countries in the world; California and Florida are the predominant strawberry-producing states, but most other states, including Iowa, have locally based production.

Anthracnose fruit rot is a major disease of strawberry that causes huge financial losses worldwide. Although we have emphasized a single fungal species – *Colletotrichum acutatum* – in the case study, strawberry is attacked by three *Colletotrichum* species: *Colletotrichum acutatum, C. gloeosporioides* and *C. fragariae*. However, *C. acutatum* is the dominant species causing anthracnose fruit rot; the other two species attack plant parts other than the fruit (Smith, 2008). *C. acutatum* and *C. gloeosporioides* are now considered a species complex (Damm et al. 2012, Weir et al. 2012). Pending more biological, ecological and pathological evidence, the anthracnose fruit rot pathogen is referred to as *C. acutatum* in this case study.

The disease triangle: a basic principle of plant pathology

The disease triangle (Figure 1) is a simple idea but an important one, because it is the foundation for the study of plant diseases: the existence of a disease caused by a pathogen (whether a fungus, bacterium, virus, etc.) requires the interaction of three

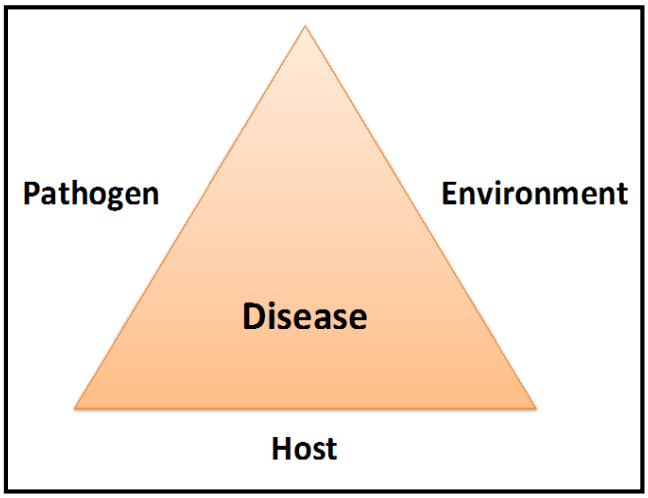

Figure 1. The disease triangle

things: a susceptible host plant, a pathogen that is capable of attacking that plant, and environmental conditions that favor the development of the particular disease. Each side of the triangle represents one of the disease components. For growers, one of the values of the disease triangle is that it helps them remember that there are three potential ways to manage a crop disease problem: suppressing the pathogen (by spraying fungicides, for example), changing the host (for example, by using varieties of crops that are genetically resistant to the pathogen), or modifying the environment (for example, by increasing plant spacing to reduce leaf wetness duration and minimize spread of the pathogen from one plant to another.)

Pathogen Biology

Colletotrichum acutatum is one of the most damaging fungal pathogens in agriculture worldwide. It has an exceptionally wide host range, including grape, blueberry, apple, almond, orange, grapefruit, lime, peach, olive, and pine as well as strawberry (Peres et al., 2005). In addition, this fungus is exceptionally adept at remaining hidden on plants for long periods of time before visible symptoms appear. Conidia, the asexual spores of *C. acutatum*, are too small to be seen individually, but can be seen easily when they are produced by the thousands in sticky, pink to orange masses on diseased fruit (Figure 2) during wet weather. Appressoria are another weapon in *Colletotrichum*'s arsenal; they are tiny survival structures that form quickly on plant surfaces and enable the fungus to withstand stresses from ultraviolet light, unfavorable temperatures, drying out, and attack by other microorganisms.

Figure 2. *C. acutatum* on strawberry fruit

Disease ecology

Contaminated nursery plants are the most important source of *C. acutatum* in commercial strawberry fields. By this means, the fungus can move hundreds or thousands of miles per day, attached to small transplants in air-mailed cartons. When *C. acutatum*-infected plants are established in a new field, conidia can germinate and infect fruit under warm, rainy conditions. From the infected fruit in the field, abundant conidia can be produced from an acervulus (a specialized spore-producing structure) and spread to healthy plants by splashing water. Even before symptoms show up on the fruit, conidia can be produced on the leaf surface and spread throughout the field, infecting new plants but initially without showing any symptoms. This "stealth" method of invisible spread makes anthracnose fruit rot especially challenging to detect and manage before the problem blows up into a fruit rot epidemic (Figure 3).

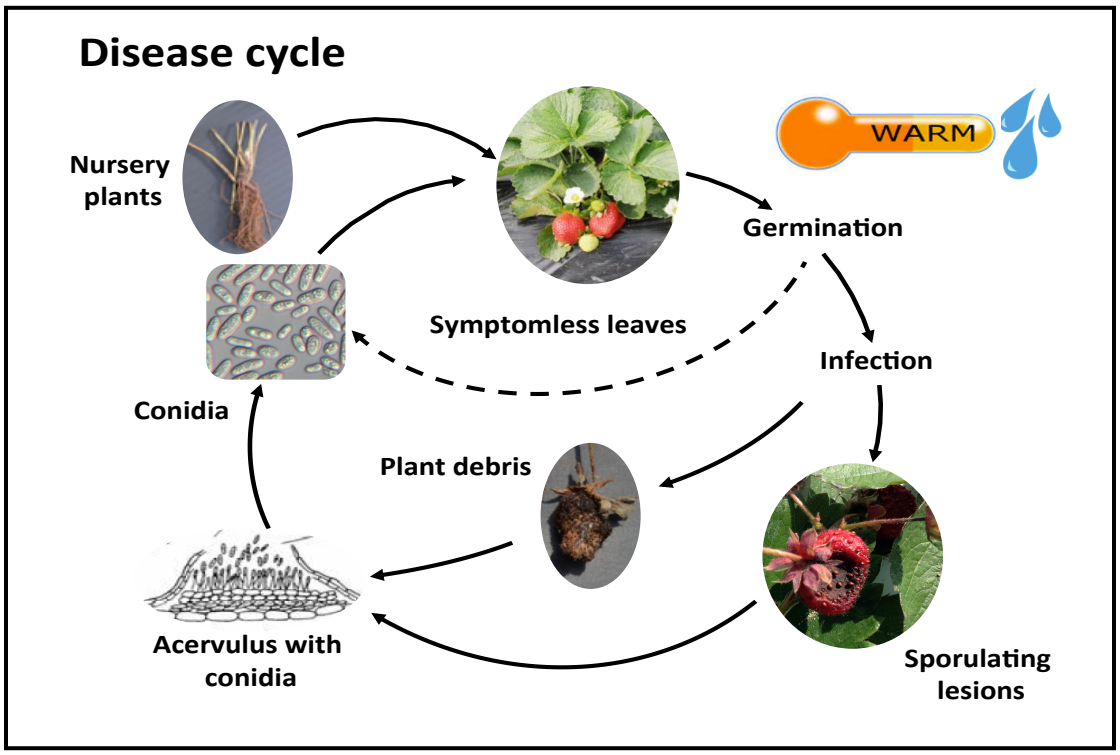

Figure 3. Disease cycle of anthracnose fruit rot caused by *C. acutatum*

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Instructor Answer Key

PART I QUESTIONS:

6. What characteristics of the fungus *Colletotrichum acutatum* can cause anthracnose fruit rot (AFR) to appear so suddenly in a field?

ANSWER: Microscopic spores (conidia) of the pathogen can stick to nursery plants and hang around on plant leaves and stems for a long time without showing any symptoms, so the fungus can spread widely in a strawberry field without being noticed. When fruit ripen, the fungus can suddenly cause sunken, brown spots that ruin the fruit.

7. What are the roles of conidia and appressoria in spreading the disease?

ANSWER: Conidia are the asexual spores of the fungus. They can multiply and spread in splashing water and infect new plants. Appressoria are survival structures that form quickly on plant surfaces from germinated conidia and enable the fungus to withstand stresses from ultraviolet light, unfavorable temperatures, drying out, and attack by other microorganisms.

8. What are suitable weather conditions for AFR to develop?

ANSWER: Warm and rainy weather between the flowering period and the start of harvest favors outbreaks of AFR.

9. What strategies is Jack currently using for AFR control (including fungicides and all the cultural practices)? How does each of these strategies reduce the threat of an AFR outbreak?

ANSWER: Jack uses the following methods for AFR control: 1) fungicide spraying, which can kill *C. acutatum*; 2) rotating individual fields out of strawberries for 3 years, which will lessen survival of the fungus in that field and thereby help reduce the risk of transmitting AFR from one strawberry planting to a later one; and 3) placing a layer of straw mulch around the strawberry plants to reduce the risk of the pathogen splashing from plant to plant.

5. a. What is the net income of Sunny Patch Farm when Jack has 70% of his field for U-Pick and 30% for pre-picked strawberry?

ANSWER:

Net income ($ per acre) = (70% U-pick net income + 30% Pre-picked net income)

$= 0.7 \times (10{,}000 - 4{,}000 - 1{,}000 - 1{,}000 - 110) + 0.3 \times (12{,}000 - 6{,}000 - 1{,}000 - 1{,}200 - 110)$

$= 2{,}723 + 1{,}107 = 3{,}830$

Net income whole farm ($) $= 3{,}830 \times 15 = 57{,}450$

b. What is the net income of jack's farm when it has 70% yield loss due to an AFR outbreak?

ANSWER:

Net income ($ per acre) = (100% - 70%) × (70% Gross income of U-pick + 30% Gross income of Pre-picked) - 70% (50% Labor of U-pick + other cost) - 30% (50% Labor of Pre-picked + other cost)

$= (1-0.7) \times (0.7 \times 10{,}000 + 0.3 \times 12{,}000) - 0.7 \times (0.5 \times 4{,}000 + 1{,}000 + 1{,}000 + 110) - 0.3 \times (0.5 \times 6{,}000 + 1{,}000 + 1{,}200 + 110)$

$= 3{,}180 - 2{,}877 - 1{,}593 = -1{,}290$

Net income whole farm ($) $= -1{,}290 \times 15 = -19{,}350$

PART II QUESTIONS:

1. Describe how the AFR warning system works.

ANSWER: The AFR warning system inputs measurements of wetness duration (total number of hours per day that the plants are wet) and air temperature, collected on the farm, to calculate the risk of an AFR outbreak, using an equation developed from studies of *C. acutatum* biology. The weather data can be collected with a commercial weather station that includes sensors to track the weather conditions and a datalogger to record the weather data. A built-in computer chip runs the equation, converting the weather data into an AFR risk rating and issuing a recommendation each day about whether or not to apply a fungicide spray.

2. Based on the disease triangle concept:

    e. Are there strategies that Jack could use to keep *C. acutatum* from getting into his fields?

ANSWER: the AFR pathogen gets into a field initially by attaching to nursery plants without causing symptoms. So Jack could quarantine the shipments of the nursery plants he received, then apply a trustworthy detection method for *C. acutatum* (several DNA-based tests have been used experimentally), and refuse to accept delivery if any shipments test positive for this fungus. He also can choose strawberry varieties that are resistant to AFR.

    f. If the fungi were confirmed NOT to be present in the field, would it make sense to spray fungicides against AFR anyway? Why or why not?

ANSWER: If tests do not detect the pathogen in a production field, it is likely that AFR won't develop there, since one component of the Disease Triangle – the pathogen - is absent. In that case, fungicide sprays would not be needed. But suppose the pathogen WAS present in the field, but at too low a concentration for testing to detect – in other words, below the limit of detection

of his test method; should Jack still spray fungicide even though his testing results were negative? Additional factors that may affect Jack's decision include the degree to which the current weather conditions favor AFR outbreaks, the level of genetic resistance of his strawberry varieties to AFR, and his customers' reluctance to buy heavily sprayed strawberries.

3. Based on the information presented in the case:

    a. Would you use the warning system if you were Jack O'Neil?

ANSWER: You could use the warning system to reduce fungicide sprays for the following reasons: meet your customers' demands for reducing pesticide use; save some money on sprays (about $40 per acre per spray); reduce the risk of fungicide resistance; and protect the environment on your farm by reducing fungicide exposure of pesticide applicators, consumers, and other organisms (for example, pollinators, natural enemies of pest insects, fish, and soil microorganisms).

    b. If yes, how can he make sure that the warning system is working in his field?

ANSWER: Since using the warning system means a significant change in the way Jack manages the AFR threat, it would be a good idea to start small. Trying out the operation of the warning system on a small field for a few years would help him to gain confidence in the new system without risking large losses. If the system works reliably for a few years on the small field, its use can be expanded to more production fields. To meet customers' demands, Jack could first expand the warning system to pick-your-own strawberry fields (where customers have frequent contact with the plants and fruit) and later to pre-picked fields.

What are possible problems associated with using it?

ANSWER: There are two main risks associated with using the warning system: 1) malfunctions with the weather-monitoring equipment could cause you to miss a critical period for applying a fungicide spray; and 2) prolonged rainy weather can make it difficult to respond to a spray warning in a timely way, even after a warning has been issued.

What additional information would be useful to help you decide?

ANSWER: Before applying the warning system in his farm, he needs to know: 1) how many sprays, on average, he can expect to save with the warning system; 2) whether the weather monitoring system is reliable; 3) where to set up the equipment to collect the weather data on his farm; and 4) how much money he can expect to save (including labor for each management option).

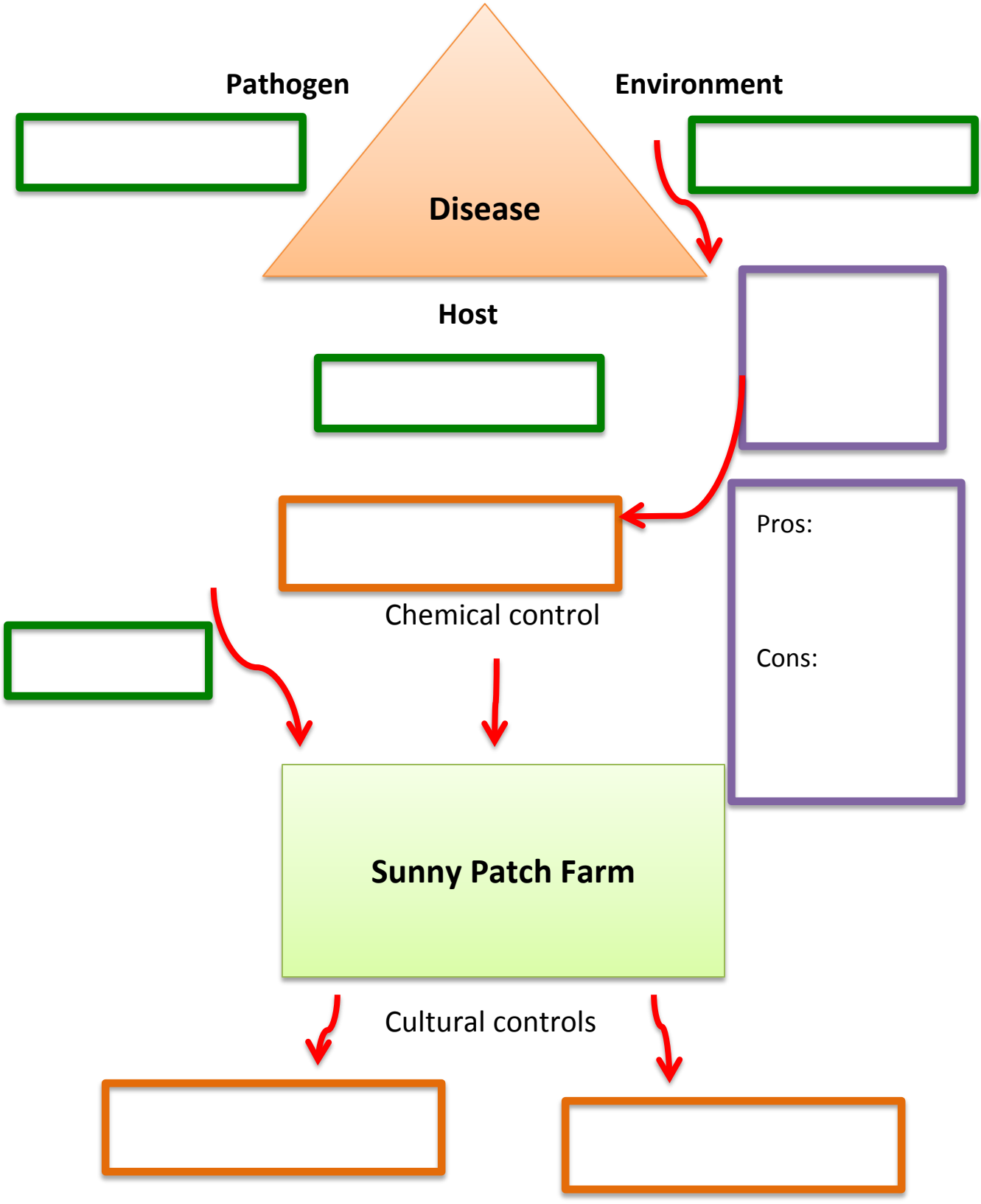
c. How will using the warning system help Jack to reduce the risk of resistance development?

ANSWER: A disease-warning system can save fungicide sprays, which reduces the risk of resistance development because it lessens the exposure of the fungus to the fungicide. In turn, less exposure means less selective pressure that would favor the predominance of resistant individuals of *C. acutatum* on the farm.

d. If Jack decides not to use the warning system, how can he meet his customers' demands that he cut back on fungicide use?

ANSWER: If he decides not to use the warning system, Jack can try alternative tactics to reduce the risk of AFR. If he uses AFR-resistant varieties, he could potentially need fewer fungicide sprays. But will his customers like the berries from the resistant varieties as well as those from the susceptible varieties he currently plants? Will they grow as well on his farm as the varieties he now grows?

Mind map for class use



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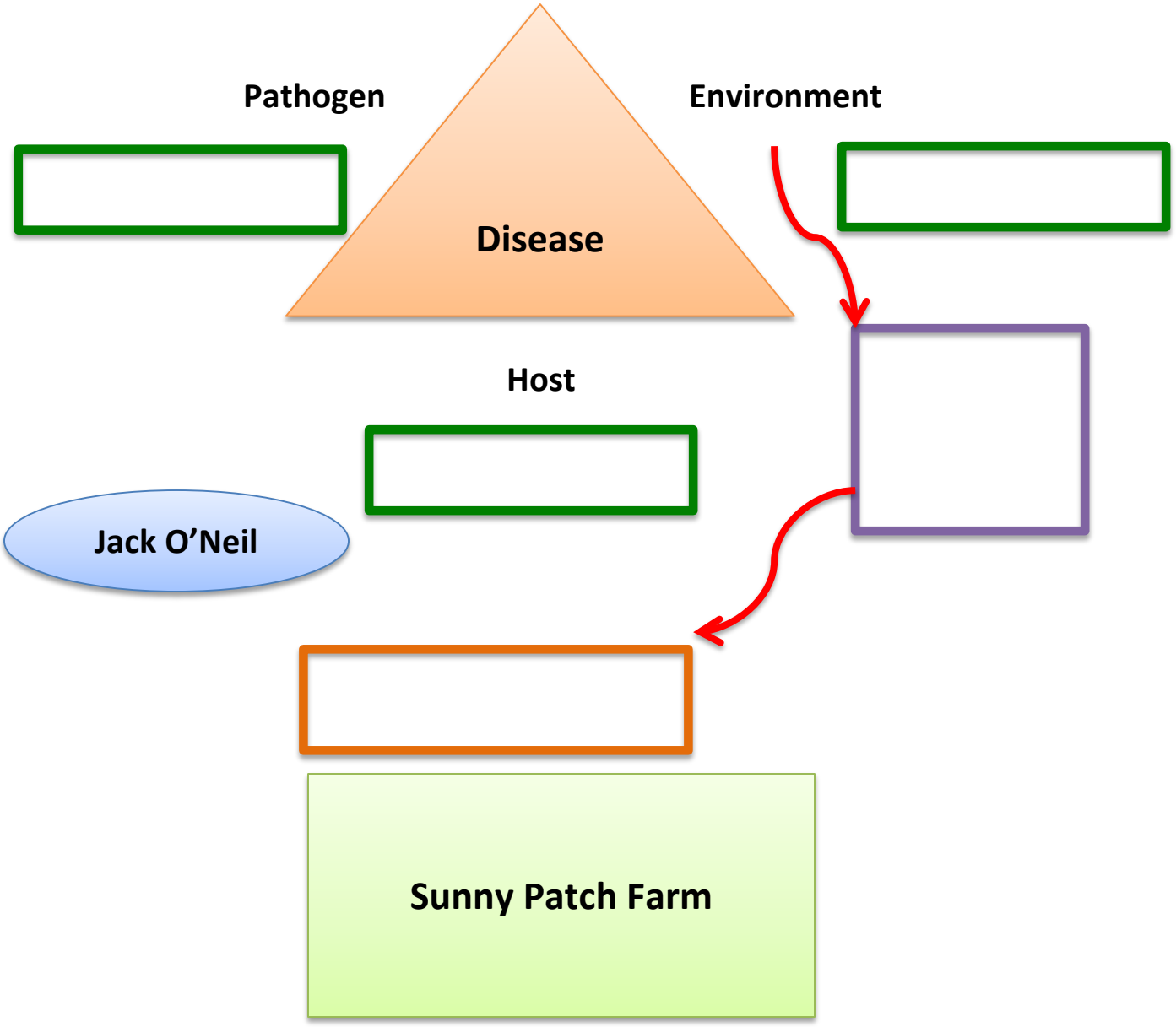
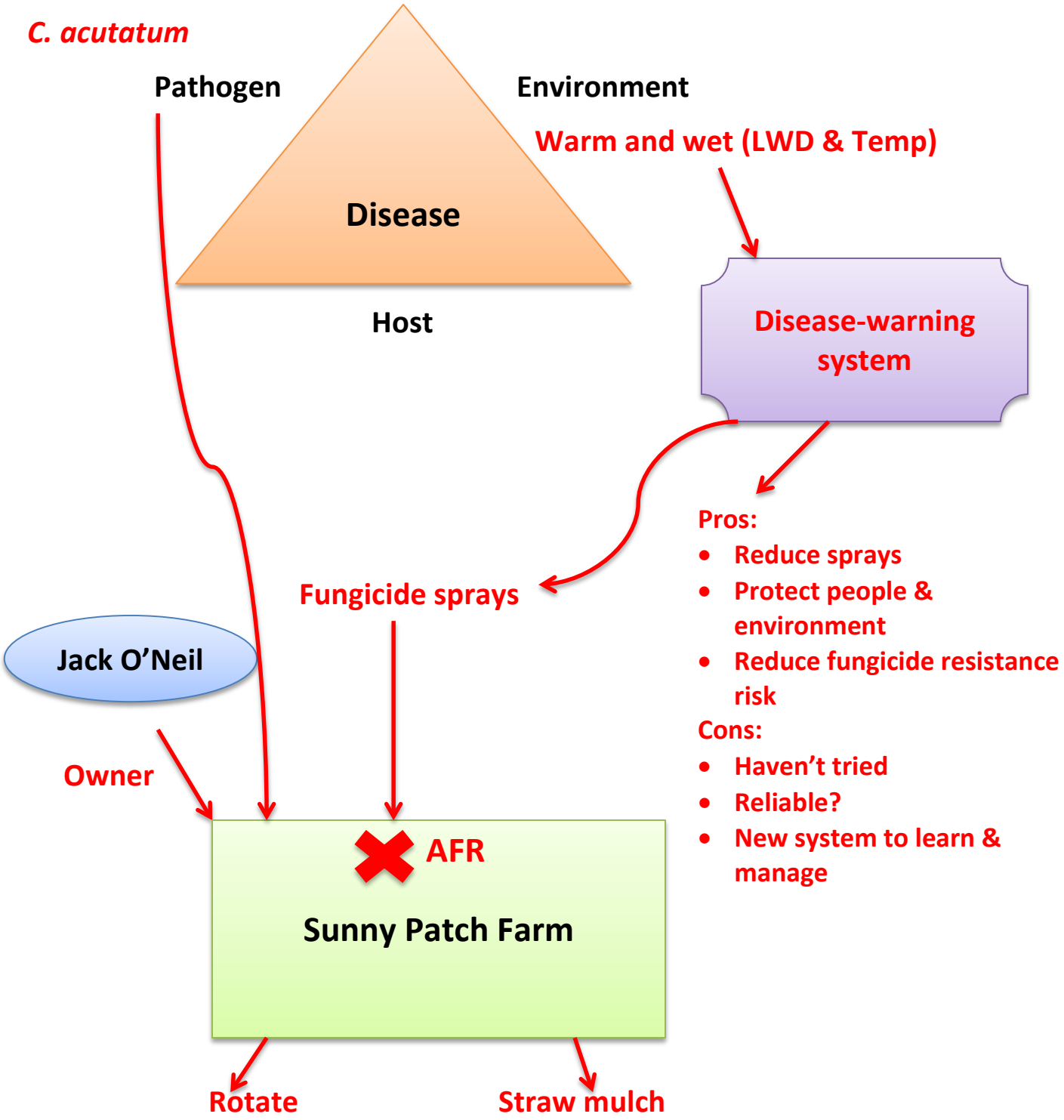


Mind map for instructor use



CHAPTER 5. GENERAL CONCLUSIONS

Colletotrichum acutatum sensu lato is the primary causal agent of strawberry anthracnose fruit rot (AFR). Management of this disease includes a key challenge of detecting the pathogen on asymptomatic plants. This research developed a loop-mediated isothermal amplification (LAMP) assay that incorporated two sets of primers targeted on the ITS region of ribosomal DNA and the β-tubulin 2 gene respectively. The LAMP assay was proven to be an effective detection method with samples from pure culture, greenhouse plants, and field plants.

A field experiment at the ISU Horticulture Research Station near Gilbert, IA was conducted during 2012-2014 to validate an AFR warning system that was previously developed and tested in Florida. In each testing year, the AFR warning system saved one to two fungicide sprays compared to calendar-based treatments. In general, the warning system-based treatments controlled AFR as well as calendar-based sprays. The results provide evidence that the Florida warning system may be valuable for helping Midwest strawberry growers reduce fungicide use against AFR.

Finally, a case study was developed to help students understand how plants become infected and how a disease-warning system uses information about the weather to help growers manage diseases with less reliance on fungicides. The case was used in classes related to horticulture and plant pathology at Iowa State University, and will be available online for educators and students.